ANALYSIS OF GENE EXPRESSION IN ENDOSULFAN EXPOSED HOMARUS AMERICANUS LARVAE USING AN OLIGONUCLEOTIDE MICROARRAY

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ABSTRACT

Potato farming and lobster fishing are two important industries on Prince Edward Island. However for both industries to be sustainable, they must be managed responsibly. Recently, agricultural runoff has been suspected to have caused numerous fish kills in rivers throughout the island. Although a pesticide research and monitoring program was implemented for freshwater areas in Canada by Environment Canada, there is insufficient information about pesticide levels and impacts in estuarine and marine environments. One agricultural pesticide of concern is the organochlorine endosulfan that is used to combat the Colorado potato beetle. Endosulfan is a potent neurotoxin and moult inhibitor that can have serious effects on non-target organisms such as the larvae of the American lobster, Homarus americanus.

The lobster life cycle consists of a larval pelagic phase followed by the migration of postlarvae to a benthic habitat. For the lobster to undergo normal development, they must moult their hardened exoskeleton to allow for growth and tissue expansion. One specific moult event is linked to the crucial developmental stage of metamorphosis, wherein the lobster transitions from a larva to a postlarva. Metamorphosis is a sensitive developmental period during which the lobster experiences significant morphological, physiological, biochemical, behavioural and ecological changes. Since this is a critical stage of development, exposure to endosulfan could have deleterious effects on development and survival of lobster larvae.

The focus of this study was to determine the effects of environmentally relevant concentrations of endosulfan on gene expression during metamorphosis of lobster larvae. Endosulfan causes serious developmental delays and deformities in an array of

species, however very little is known about the regulation of gene expression during pesticide exposure. The use of a custom made high throughput lobster, H. americanus, microarray allowed for monitoring of 14,592 genes based on unique lobster expressed sequence tags (EST). A pooled reference design was used to identify changes in gene expression between 5 endosulfan concentrations and a control. Genes with >1.5 fold change and identified as being significant at $p < 0.05$ using one-way ANOVA were selected for further analysis. There were 707 genes identified as being significantly differentiated. However with only ~40% annotation of the array, the majority of these genes were unknown. Annotated genes were involved in many processes: development, metabolism, immune and oxidative stress response and gene regulation.

Nine genes of interest (GOI) were selected for reverse transcription quantitative PCR (RT-qPCR) analysis to validate the microarray results. For optimal RT-qPCR normalization, 5 housekeeping genes were identified and validated using geNorm. Although the RT-qPCR detected a similar expression pattern as the microarray, the microarray results were often greatly under expressed. Due to discrepancies between expression levels in the microarray compared to the RT-qPCR method, the correlation values between the two were low.

Endosulfan had a serious effect on survival, development and gene expression during metamorphosis. The long term objective of this research will be to use microarray gene expression profiles as screening tools for identifying what contaminants are present in the environment.

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DEDICATION

I would not be where I am today is it was not for my family. To my parents, your unconditional; love and support has been instrumental throughout all aspects of my life. You have supported and helped me achieve my goals, many of which I was convinced were unnatainable. To Grampy, my greatest supporter, although you could not be beside me when I completed my project, I know you were watching over me.

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LIST OF ABBREVIATIONS

3DE	3-dehydroecdysone
20E	20-hydroxyecdysone
25dE	25-deoxyecdysone
a.i.	Active ingredient
ALET	Atlantic Laboratory for Environmental Testing
Bref	Brefeldin A
CYP 450	Cytochrome P450
Ca^{2+}	Calcium ion
CCME	Canadian Council of Ministers of the Environment
cDNA	Complementary deoxyribonucleic acid
CHP	Conserved hypothetical protein
CF	Calibration factor
CFIA	Canadian Food Inspection Agency
CHH	Crustacean hyperglycaemic hormone
CNRQ	Calibrated normalized relative quantities
Ct	Critical threshold
CZRI	Coast Zones Research Institute
DDT	Dichlorodiphenyltrichloroethane
DFO	Department of Fisheries and Oceans
DCM	Dichloromethane
dNTP	Deoxyribonucleotide triphosphate
DO	Dissolved oxygen
DHR3	Drosophila hormone receptor 3
DTT	Dithiothreitol
EIP-75	Ecdysone inducible protein
EC_{50}	Median effective concentration
EDC	Endocrine disrupting chemical
EDTA	Ethylenediaminetetracetic acid
EST	Expressed Sequence Tag
ETC	Electron transport chain
EtOH	Ethanol
FA	Farnesoic acid
FAO-MeT	Farnesoic acid o-methyltransferase
FTZ-F1	Fushi tarazu F1
Fe	Iron
g	Relative centrifugal force
GABA	Gamma-aminobutyric acid
GAL	GenePix array file
GAPS	Global atmospheric passive sampling
GC-ECD	Gas liquid chromatography dual electron capture detector
GH	Glycoside hydrolase
GFP	Green fluorescent protein
GPC	Gel permeation chromatography
GPx	Glutathione peroxidase

GO	Gene ontology
GOI	Genes of interest
GST	Glutathione-S-transferase
ha	Hectare
HSP	Heat shock protein
Hyploc	Hypothetical protein
JH	Juvenile hormone
L	Litre
LC_{50}	Median lethal concentration
LDH	Lactate dehydrogenase
LFA	Lobster fishing area
LLCP	Live lobster certification protocol
Log K_{ow}	Log Octanol-Water partitioning coefficient
LOWESS	Locally weighted scatter plot smoothing
IDT	Integrated DNA Technologies
IRC	Inter run calibrators
m	Metre
MDL	Minimum detection limit
MOA	Mode of action
MEGA	Molecular Evolutionary Genetics Analysis
MF	Methyl farnesoate
MIAME	Minimum information about a microarray experiment
MIH	Moult inhibiting hormone
MIQE	Minimum information for publication of quantitative real-time PCR experiments
mm	Millimetre
MO	Mandibular organ
NB	New Brunswick
NF	Normalization factor
NOEC	No observed effect concentration
NRQ	Normalized relative quantities
PEI	Prince Edward Island
PCB	Polychlorinated biphenol
PGC	Primordial germ cells
PMRA	Pest Management Regulatory Agency
PMT	Photomultiplier tube
POP	Persistent organic pollutants
proPO	Prophenoloxidase
ON	Ontario
ROS	Reactive oxygen species
RQI	RNA quality indicator
RXR	Retinoid X receptor
RT	Reverse transcription
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SDS	Sodium dodecyl sulfate
SO_2	Sulfur dioxide
SOP	Standard operating procedure

SR/ER	Sarcoplasmic reticulum/ Endoplasmic reticulum
SSC	Saline sodium citrate
SULT	Sulfotransferase
TBE	Tris Borate EDTA buffer
TIFF	Tagged image file format
Tm	Melting temperature
UGT	Uridine diphospho glucuronsoysltransferase
UNEP	United Nations Environment Program
USA	United States of America
US EPA	United States Environmental Protection Agency
USP	Ultraspiracle
Wg	Wingless
WP	Wettable Powder

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Chapter 1: GENERAL INTRODUCTION

1.1 Importance of the lobster industry

The American lobster (H. americanus) is found primarily on the east coast of North America ranging from North Carolina to Newfoundland at depths of 50 m to more than 700 m (Cobb and Castro, 2006; Wahle and Fogarty, 2006). Although sub-tidal locations such as the Gulf of St. Lawrence, along the southern shores of Nova Scotia and the Gulf of Maine are the more productive areas, there are also deep water populations along the continental shelf (Aiken and Waddy, 1986; Factor, 1995). The lobster fishery is a corner stone for the economy in Atlantic Canada and Québec accounting for annual export sales of approximately $800 million dollars in 2009 (DFO, 2011). Recently the industry has suffered from the global economic crisis, the strength of the Canadian dollar and increased production costs (Gardner Pinfold Consulting Economist Limited, 2006). Despite the economic turmoil, the American lobster is the most valuable and widely commercially distributed marine species from Atlantic Canada and is exported to 59 countries (DFO, 2011). Most of the lobster destined for the international market will be sold in the United States of America (USA) accounting for 80% of export goods and exemplifying why the industry is vulnerable to American prices and the fluctuating U.S. dollar (Gardner Pinfold Consulting Economist Limited, 2006; DFO, 2011).

In 2008 there were 10,000 lobster fishing licenses issued and the industry was estimated to employ 25,000 to 35,000 people, primarily in rural and coastal communities (DFO, 2011). Fishermen in the Atlantic Provinces and Québec must apply to receive a federal government issued fishing license for one of the 41 lobster fishing areas (LFA) in the region. The lobster industry is heavily regulated by the federal government,

particularly by the Department of Fisheries and Oceans (DFO) and the Canadian Food Inspection Agency (CFIA). The goal of government issued regulations is to maintain a sustainable lobster population while also delivering high quality product to the consumer.

Most fishing seasons in the LFAs are designated throughout the spring and fall in an attempt to avoid the summer moults and to catch larger, more mature individuals (DFO, 2011). A large percentage of the lobster catch is harvested off the Scotian Shelf and from the Gulf of St. Lawrence around Prince Edward Island and New Brunswick (DFO, 2011). DFO has established a minimum carapace length of 71-84 mm, depending on the LFA, a maximum carapace length of 127 mm and prohibited the capture of egg bearing females to ensure that the reproduction cycle can continue. Given that the lobster industry is such a valuable resource in Atlantic Canada, the management of the lobster population is vital to maintain a sustainable fishery.

Exporters who ship their product nationally or internationally must be certified by CFIA and meet the standards identified in the Fish Inspection Regulations. Both registered and non-registered exporters must submit live lobster certification protocols (LLCP) that contain relevant background information, hazard analysis, monitoring and preventative measures, a sanitation program and a detailed tracking system of shipped goods (CFIA, 2012).

1.2 Lobster biology

The American lobster, Homarus americanus, is a member of the subphylum Crustacea and the order Decapoda. The body structure of crustaceans is divided into three regions: the head, thorax and abdomen. In the lobster, the head and thorax are fused together forming the cephalothorax and is covered with an exoskeleton or carapace. The carapace is a hardened shell that functions as protection and is composed of calcium, magnesium, phosphate, carbonate and α-chitin (Boβelmann et al., 2007). The hardness of the exoskeleton is not uniform and is dependent on the structure function relationship of the body part allowing for both protection and flexibility (Boβelmann et al., 2007). Lobsters are classified as decapods because of the five pairs of pereiopods or "walking legs" found on the posterior region of the cephalothorax, that are used for locomotion. The first set of pereiopods (chelipeds), also known as the crusher and cutter claws, are considerably larger than the other pereiopods and are important for feeding behaviours and determining male dominance (Factor, 1995; Lawton and Lavalli, 1995). The abdomen contains the uropods (telson), reproductive organs and pleopods or "swimmerets" that are used for swimming in mature lobster.

Lobsters are dioecious, where male and female can be identified by the differences in the structure and function of several anatomical features. The first set of pleopods on a male are modified for mating and spermatophore transfer, whereas these pleopods are under developed and vestigial in the female (Factor, 1995; Talbot and Helluy, 1995). Males also possess a longer carapace, while females have a wider abdomen and tail to support the eggs carried on her pleopods after extrusion (Gosselin et al., 2003; Talbot and Helluy, 1995). In addition to the wider abdomen, sexually mature

females have seta projections on their pleopods to which extruded fertilized eggs are attached (Talbot and Helluy, 1995).

1.3 Spawning

Spawning of fertilized eggs occurs at different times depending on geographic locations and water temperature, however, most spawning events occur during the summer months of June-August (Aiken and Waddy, 1986; Templeman, 1940). Inshore lobster populations moult and spawn in early summer, whereas offshore moulting and spawning cycles occur later in the summer or into September (Aiken and Waddy, 1986). Females must not be disturbed during the extrusion of the eggs as it may result in the loss of the brood. External brooding is risky and if the embryos are to survive the 9-16 month incubation period, several conditions must be met (Talbot and Helluy, 1995; Waddy et al., 1995). The embryos must have sufficient yolk as a source of nutrients, be securely attached to the pleopods and have limited exposure to pathogens and unfavourable water conditions (Aiken and Waddy, 1986). For each brood between 30-50% of each clutch is lost naturally as a result of parasite infection, maintenance cleaning by the female, poor attachment or by handling of trapped females (Talbot and Harper, 1984; Talbot and Helluy, 1995).

1.4 Larval development

Research into lobster larval development is increasing; however it continues to lag behind information on juvenile and mature crustaceans. The study of crustacean larval development is challenging because of the difficulty in rearing larvae in suitable

representative environments, the inability to perform biological assays on a single individual due to their small size and the difficulty in collecting and staging wild larval individuals (Anger, 2006). Consequently, most of what has been inferred about larval development is often a projection of the research done on mature adults. Understanding how larvae progress through development is important for our understanding of the complex crustacean life cycle.

During a period of 9-16 months, fertilized eggs undergo embryonic development into nauplii, while maintaining attachment to the female lobster's pleopods (Helluy and Beltz, 1991; Talbot and Helluy, 1995). Many benthic and invertebrate species have a complex life cycle transitioning through embryonic, larval and postlarval stages of development as opposed to development from the egg to an immature adult (Anger, 2006). During the summer months, between May and September, the nauplii hatch becoming free swimming larvae. Homarus americanus larvae develop through three planktonic larval stages, I, II & III during a 6-8 week period (Ennis, 1995). In rearing larvae under laboratory conditions at 15 °C, the average number of days per stage was 4.5, 5.5 and 8, respectively (Chang et al., 2001). The description of H. americanus larvae was first documented by S. Smith in 1873 and although morphologically stages I-III appear similar, they can be identified by their increasing size and changes in anatomical characteristics (Charmantier et al., 1991; Factor, 1995; Herrick, 1911).

Stage I larvae are found in the pelagic zone and are identified by the presence of exopodites on the maxillipeds and pereiopods that function in locomotion. Their body structure is curved and they lack both pleopods and uropods (Factor, 1995). Stage II larvae are anatomically similar to stage I; however they exhibit initial development of pleopods on the abdomen and a reduction in the size of the exopodites. Stage III larvae

are the largest of the larval stages and have fully functional emerged pleopods and uropods for locomotion, with reduced and nonfunctional exopodites.

All three larval stages are opportunistic feeders on suspended planktonic matter such as zooplankton and small crustaceans (Fiore and Tlusty, 2005). The larval diet must maintain a balance of protein, lipids and to a lesser extent, carbohydrates to accommodate the rapid growth that occurs during these stages (Capuzzo and Lancaster, 1979; Sasaki, 1984; Sasaki et al., 1986). The growth rate of planktonic larvae are nearly double that of benthic postlarvae resulting in greater daily energy intake (Sasaki, 1984; Wahle and Fogarty, 2006). Larvae do not have sufficient energy stores and rely heavily on available lipid and protein in their diet to sustain a high growth rate (Sasaki, 1984; Sasaki et al., 1986). Biochemical profiles are different between stages and throughout the moult cycle when physiological and morphological changes require different allocation of energy and metabolites (Sasaki et al., 1986). The diet during the larval stages is important for normal development; negative dietary conditions will affect the development and survival of the postlarvae (Fiore and Tlusty, 2005).

1.5 Metamorphosis

In the American lobster, metamorphosis is achieved after the third moult, where the larva has developed from a stage III to a stage IV postlarva (Charmantier et al., 1991). Metamorphosis is considered a defining stage in development where larval characteristics are lost and the organism begins to resemble the adult, yet maintains some characteristics that are different from both the larval and mature stages (Charmantier et al., 1991). Initially, metamorphosis was identified by morphological

changes; however, the definition has evolved to include changes in physiology, ecology and behaviour (Charmantier et al., 1991). Significant physical changes that occur in H. americanus during metamorphosis are increased development of the pleopods, increased size of the chelipeds and a more streamlined body plan. Locomotion becomes increasingly important as the postlarvae begin to move to a benthic environment in search of a protective habitat.

With increased ability to move within the water column and benthic environment, postlarvae can pursue different food sources. All life stages of the lobster prefer live food; however the active pursuit of small crustaceans (including other lobsters) and insects begins as postlarvae. Despite the newly developed feeding behaviour of postlarvae, they continue to be opportunistic feeders to meet their nutritional requirements (Ennis, 1995). As the diet of the developing lobster shifts after metamorphosis, so does the bioenergetic distribution. Postlarvae show slight decreases in the percent of protein in their diet and increased lipid consumption, suggestive of a reliance on lipid stores once occupying a more cryptic habitat (Ennis, 1995; Sasaki, 1984; Sasaki et al., 1986). Throughout larval and juvenile development, the lobster will consume 10% of its weight/day compared to the minimal 1% consumed by adults (D'Abramo and Conklin, 1985).

1.6 Moult regulation and the endocrine system

Growth rate is defined as an increase in size over time, where growth in crustaceans is achieved through moulting (Cobb and Castro, 2006). Lobsters escape their exoskeleton for a newly synthesized carapace that will allow them to increase in

size and is necessary for growth, development and reproduction (Chang, 1995; DFO, 2011). The term moult refers to all the physiological, morphological, biochemical and behavioural changes that are involved in the shedding of the exoskeleton allowing the lobster to increase in volume up to 50% (Waddy et al., 1995). The moulting process is an example of discontinuous growth where the lobster passes through different identifiable stages to reach sexual maturity and continues through normal development (Lachaise et al., 1993). The growth pattern results in the classification of individuals into post-moult, intermoult and pre-moult lobsters (Lachaise et al., 1993). The moult cycle is complex and involves the interaction of many biological and physical processes over the course of days to months (Chang, 1995). The act of shedding the protective carapace (ecdysis) must happen quickly to reduce the risk of exposure to predators. The larval moulting cycle is difficult to study effectively in the wild and it is assumed that known physiological and biochemical processes that occur in juvenile and adults also occur in larvae (Anger, 2001; Anger, 2006; Charmantier and Charmantier-Daures, 2001).

The transition from larval to postlarval stage is controlled by the endocrine system. Changes in the expression of target genes are initiated by the binding of ligands to nuclear receptors that subsequently bind to DNA resulting in the initiation or inhibition of transcription (McWilliam and Phillips, 2007). There is considerable research into understanding the molecular mechanisms behind metamorphosis in insects and apparently the process is similar in crustaceans (Deng et al., 2011; McWilliam and Phillips, 2007).

Within the moult cycle, each phase is sub-divided into stages classified A through D (Waddy et al., 1995). After the lobster has shed its exoskeleton, the newly

exposed cuticle is thin and weak, signifying the beginning of post-moult stage A. During the moult, calcium stores from the gastroliths in the stomach are released to ensure that the parts of the body needed for foraging such as the chelipeds, maxillipeds, mandibles and teeth are hardened quickly (Waddy et al., 1995). Ingestion of the newly shed exoskeleton also provides valuable nutrients used in calcification of the hardening carapace.

Twenty-four to forty-eight hours after moulting, the endocuticle lamella will form indicating the transition from Stage A to Stage B (Waddy et al., 1995). As the cuticle continues to harden through calcification, the lobster will enter into Stage C, which is divided into 4 sub-stages. After the lobster has reached Stage C_4 it has completed postmoult and entered into intermoult, signifying the completion of the new cuticle. The progression through postmoult can last up to 65% of the moult cycle (Waddy et al., 1995). Premoult is identified as Stages D_0-D_3 where the new cuticle begins to form under the existing exoskeleton (Comeau and Savoie, 2001; Lachaise et al., 1993; Waddy et al., 1995). As the lobster prepares to moult, it enters into stage D_0 of the premoult phase where the epidermis begins to separate from the cuticle (Waddy et al., 1995). Stage D_0 is described as a transition stage where growth can cease until environmental conditions are optimal for development to continue (Waddy et al., 1995). When appropriate, the moult cycle will resume and the lobster will begin secreting a new cuticle throughout stages D_1 to D_3. During the final premoult D_4 stage, the lobster ingests water that is absorbed by the lining of the digestive tract (Mykles, 1980). The increased fluid in the hemolymph causes the carapace to rupture, allowing ecdysis to occur. The frequency of moult cycles is highly dependent on water and environmental

conditions. Juvenile lobsters can moult 3 or 4 times in a year, whereas mature lobster may only moult once every year or two (DFO, 2001).

In insects, moulting is regulated by the steroid hormone 20-hydroxyecdysone (20E) (commonly known as ecdysone) and the juvenile hormone (JH). JH is not found in crustaceans, however methyl farnesoate (MF) is hypothesized as being the equivalent to the insect JH and involved in metamorphosis and reproductive development in crustaceans (Abdu et al., 1998; Borst et al., 1987; 1998; Chang et al., 2001; Laufer and Biggers, 2001). Ecdysone is also found in crustaceans and was initially called crustecdysone, but was later discovered to be the same moult inducing agent as in insects and thus the name ecdysone was retained (Chang, 1995). Ecdysteroids are polyhydrated steroids synthesized from cholesterol in the process of ecdysteroidogenesis (Lachaise et al., 1993). Ecdysteroidogenesis occurs in ectodermally derived Y-organ or the moulting gland that is located bilaterally in the anterolateral region of the cephalothorax (Waddy et al., 1995). The most common ecdysteroid is 20-hydroxyecdysone (20E). Research on various crab species has identified two other ecdysteroids, 3-dehydroecdysone (3-DE) and 25-deoxyecdysone (25-dE) that are likely involved in the moult cycle (Chang et al., 2001; Lachaise et al., 1989; Spaziani et al., 1989). 3-DE has been identified as the secreted form of the moult inducing hormone which is converted to ecdysone and later to the active form of 20E in peripheral tissues (Lachaise et al., 1993; Tiu et al., 2010).

The level of ecdysteroids in hemolymph depends on the moult stage and sub-stage (A, B, C or D). A lobster in postmoult and intermoult will have low levels of ecdysteroids until premoult (Chang et al., 2001; Lachaise et al., 1993; Waddy et al., 1995). After the lobster has progressed into premoult D_1, ecdysteroid levels begin to

rise in preparation for the moult and decrease prior to ecdysis (Lachaise et al., 1993). Early moult experiments with the removal of the eyestalk resulted in increased moult frequency, indicating that the eyestalk had an inhibitory effect on moulting (Fingerman and Fingerman, 1976). The neuropeptide moult inhibiting hormone (MIH) was later identified and is secreted by the X-organ sinus gland complex found in the eye stalk (Lachaise et al., 1993). MIH is synthesized by neurosecretory cells in the X-organ, transported along axons to the sinus gland where it is stored until its release (Fanjul-Moles, 2006). Release of ecdysteroids into the hemolymph is negatively regulated by the neuropeptide MIH that decreases the synthesis of ecdysone from the Y-organ preventing the initiation of moult (Chung and Webster, 2003; Lachaise et al., 1993; Rangarao, 1965).

Unlike ecdysone and MIH, there is considerable uncertainty regarding the function of MF in crustaceans. MF is produced in the mandibular organ in crustaceans and is a precursor to the JH III in insects (Nagaraju, 2007). Juvenile hormone is a moult inhibitor in insects; however there is still debate as to whether MF elicits the same response in crustaceans. A study on the fiddler crab (Uca pugilator) found that injection of MF had no effect on ecdysteroid signaling (Felterman and Zou, 2011) yet there are multiple studies on H. americanus and other species that suggest MF has an effect on the transition through metamorphosis and reproductive maturity (Adbu et al., 1998; Borst et al., 1987; Laufer and Biggers, 2001; LeBlanc, 2007). Despite conflicting results on MF, it apparently functions as an endocrine signaling molecule in crustaceans. One possible conclusion for the inconsistencies in MF experiments is that MF may have multiple effects that are dependent on specific developmental stages of crustacean species (Nagaraju, 2007).

Ecdysone, MIH and MF are not the only components involved in regulation of the moult cycle. The identification of two other neuropeptides with a similar function to MIH suggests that crustacean hyperglycemic hormone (CHH) and vitellogenesis-inhibiting hormone (VIH) may also play active roles during the moult cycle (Chang, 1995). Initially, CHH was found to decrease levels of glucose in the hemolymph and was given the name crustacean hyperglycemic hormone (Abramowitz et al., 1944; Chang et al., 2001). There are two CHH isomers, CHH-A that functions on hyperglycemia and moult inhibition and CHH-B that is involved solely in hyperglycemia (Chang et al., 2001). CHH from H. americanus has shown 90% similarity to MIH (Chang, 1995). Similar to ecdysteroids, CHH titres vary throughout the moult cycle with higher levels present during late premoult (Chang et al., 2001).

1.7 Developmental stressors

Lobster larvae have to cope with many environmental and anthropogenic stresses and although certain individuals are better able to adapt than others, survival is not indicative of overall health. Some factors affecting normal development are: suitable habitat during benthic migration, temperature, salinity, food availability and quality, and anthropogenic stressors (Ennis, 1995). These factors, whether in combination or individually, can affect growth and development throughout the lobster life cycle and can have carry-over effects to the next life stage (Anger, 2006).

1.7.1 Habitat disruption

Newly emerged stage IV postlarvae, which have undergone metamorphosis, begin to gain characteristics of a mature lobster and migrate towards a benthic environment in search of a suitable habitat (Charmantier et al., 1991). If substrate conditions are unfavourable for postlarvae to settle, they continue to explore other areas. Lobsters prefer rocky habitat that allows them to live a sedentary life and provide protection against predation (Wahle and Fogarty, 2006). Cobb (1968), found that postlarvae that were presented unfavourable bottom substrate showed delays in moulting. An extended pelagic phase during the search for an appropriate benthic environment can be detrimental for the survival of postlarvae because it increases the risk of predation (Aiken and Waddy, 1986). Lobsters are selective about their habitat substrate and exhibit intraspecific aggression. Lobsters are extremely territorial and laboratory experiments exploring the effect of multiple larvae in the same habitat resulted in moult and settling delays (Cobb, 1970).

1.7.2 Temperature

Moulting of lobster larvae is highly dependent on environmental and biological conditions. Larvae develop to stage IV postlarvae sooner in warmer water temperatures of ~20 ºC which suggests that temperature is one of the most important environmental factors on growth and development (Ennis, 1995; Templeman, 1936a). Moulting can occur at temperatures slightly below 10 ºC, however it requires more time between successive moults (Aiken and Waddy, 1986; Ennis, 1995). Although moderate temperature fluctuations do not appear to have substantial effect on survival, significant

decreases in survival of stage III and IV individuals occur at colder temperatures (Ennis, 1995; MacKenzie, 1988). Larvae hatched in very low temperatures below 5º C typically die before moulting to stage V (Aiken and Waddy, 1986; Templeman, 1936a). In warmer inshore water, lobsters have a higher metabolism, and moult more frequently than those in cooler deeper waters (Aiken and Waddy, 1986). A study on the Spiny lobster (Panulirus ornatus), a warm water species, showed similar results where warmer temperature caused a decrease in the duration of the intermoult period (Jones, 2009). The effect of temperature on growth and moult cycle of both larval and adult lobster is central to their overall development.

1.7.3 Salinity

Although lobsters are typically considered marine species, they may encounter lower salinity inshore or in estuarine habitats and after heavy rainfall or run-off events (Charmantier et al., 2001). The process of osmoregulation is facilitated by the capability to properly regulate ion concentrations and enzymes responsible for ion transport within the organism (Charmantier et al., 2001). The ability of larvae to osmoregulate depends on temperature, salinity and moult and developmental stage (Ennis, 1995). Lobster larvae reared at 15-17.5 °C between 21-32 ‰ salinity demonstrated high survivability and took fewer days to reach stage IV than larvae reared at lower salinities (Charmantier et al., 2001; Templeman, 1936b). Jones (2009) also found that tropical spiny lobster (P. ornatus) that were exposed to lower salinities exhibited slower growth rates and overall decrease in size and weight. The American lobster becomes increasingly adaptable to

varying salinities after they have reached stage IV postlarvae because of anatomical and physiological changes (Charmantier et al., 2001).

1.7.4 Food supply

Like all living organisms, adequate food supply and quality are essential for lobster growth and survival. Most of the information about lobster nutrition are the results of laboratory experiments (Aiken and Waddy, 1986). Both duration and timing of starvation affected normal development of the Spider crab (Hyas araneus) (Anger, 1987). If sufficient nutrition is obtained until premoult (D_0), then moulting will proceed. However if starvation occurs during postmoult or intermoult (Stage C_4) the moult cycle can be suspended (Anger, 1987). Although there is some debate regarding the availability of wild prey throughout different regions of the American lobster habitat, each life stage requires different food sources to obtain essential nutrients (Conklin, 1995; Grabowski et al., 2009). Larvae are not active foragers; they feed on various plankton, including zooplankton, copepods, amphipods, diatoms and the larvae of other organisms suspended in the water column (Conklin, 1995). Protein and lipids are important energetic substrates for larval development; however by stage IV there is a gradual increase in lipid content, suggesting newly acquired ability for energy storage (Capuzzo et al., 1984; Sasaki 1984; Sasaki 1896). The post metamorphic stage IV is primarily benthic, yet can continue to feed on planktonic food sources until settlement in a sheltered benthic habitat. After the postlarvae have become juveniles, it is rare that they leave their shelter and therefore rely on food sources found within or passing by the shelter (Conklin, 1995). Again, although this diet is difficult to study in the wild, it is

considered to be sufficient to sustain growth and development for the first few years of benthic life. Finally, as the developing lobster becomes mature, they gradually progress into more active foraging feeding behaviours. The exact composition of their diet at this stage is geographically and seasonally dependent; the diet consists of ~ >90% animals and ~ <5% plant material (Conklin, 1995; Grabowski et al., 2009).

1.7.5 Anthropogenic stressors

Anthropogenic stressors can also impact lobster development. For the past 200 years, lobster has been an important commercially fished species and subjected to heavy exploitation (Factor, 1995). Aside from fishing, lobster can be exposed to harmful inorganic and organic pollutants from multiple anthropogenic sources and these can have devastating effects on both adults and the more vulnerable larval stages (Aiken and Waddy, 1986; Waddy et al., 1995).

Inorganic substances including sulfur dioxide (SO_2), ammonia and metals are ubiquitous; however anthropogenic activities are increasing the presence of these compounds in the environment. Emissions from coal and petroleum produce SO_2 in the atmosphere while soil runoff from agricultural sites that have applied fertilizer can cause eutrophication of nearby lakes and rivers (Chambers et al., 2001; Newman, 1998; Yu, 2005). Metals are of great concern because of their potential to bioaccumulate in marine and freshwater species and can be found in both the water column and underlying sediment. Unlike other pollutants, heavy metals are not easily broken down by oxidation or other detoxification processes in exposed organisms (Raissy et al., 2011).

Most organic pollutants are manufactured and released into the environment causing damage when present at elevated concentrations (Newman, 1998). Organic pollutants include compounds such as polynuclear aromatic hydrocarbons (PAH), and endocrine disrupting chemicals (EDC) including pharmaceuticals, many personal care products and pesticides (Newman, 1998; Yu, 2005). PAHs develop from incomplete combustion of organic material and can be formed naturally or through anthropogenic processes such as car emissions, power generation and incineration (Yu, 2005). Many PAHs in the environment are considered to be probable carcinogens and because they are not easily degraded, they are susceptible to transportation (Yu, 2005). As pharmaceutical and personal care product use are increasing, these EDC compounds disrupt normal hormonal function in non-target organisms and can have serious teratogenic effects. Agricultural practices are also another source of EDC contamination of aquatic environments through pesticide runoff and drift (Caux et al., 1996; Dunn et al., 2010). The list of pesticides, herbicides and fungicides used in agriculture is lengthy; however one family of pesticides that continues to persist in the environment despite many of its chemicals being banned worldwide are organochlorines. Organochlorines are long lasting hydrophobic compounds that bind to sediment and organic matter and occur in many marine and freshwater species, primarily in agricultural areas (Coat et al., 2006). Exposure to such environmental toxicants can have serious effects on survival and moult regulation in crustaceans (Coat et al., 2006; Derby and Capuzzo, 1984).

1.8 Potato farming and pest management

Prince Edward Island (PEI) is recognized worldwide for its production of the famous "PEI potatoes". In 2011, 34,195 ha of potatoes were planted, accounting for ~13% of all agricultural land use on the island (PEI Department of Agriculture and Forestry, 2011). In 2006, Statistics Canada reported 112,193 ha of agricultural land in PEI was treated with fertilizers, 87,699 ha with herbicides, 41,839 ha with fungicides and 40,164 ha with insecticides to help increase the productivity of crops (Statistics Canada, 2008). In addition, 680,552 kg of active ingredient (a.i.) of non-domestic pesticide was sold on PEI in 2008 (PEI Department of Environment, Energy and Forestry, 2009). Potato farming is an important industry on PEI where annual crops are valued at $200-235 million dollars, depending on current market value (PEI Department of Agriculture and Forestry, 2011). Similar to other agricultural industries, potato farming has problems and concerns. Each year, valuable potato fields are threatened by disease, potato rot and pests which can ruin entire crops. The Colorado potato beetle (Leptinotarsa decemlineata) is one of the most damaging insects to potato crops worldwide (Ferro et al., 1985). The beetle was first identified on PEI in 1883 and continues to plague potato crops (Government of New Brunswick Department of Agriculture, Aquaculture and Fisheries, 2012). Although the beetle originated in the south western United States and Mexico, it has now spread to most agricultural fields in North America, Europe and Asia (Alyokhin, 2009).

Adult beetles remain dormant during winter months and emerge in early spring, which coincides with the emerging potato plants. Females lay 300-800 eggs and after hatching, larvae will begin feeding within 24 h (Alyokhin, 2009). A single potato beetle larva can consume up to 40 cm^2 of foliage while a mature adult can consume 9.65 cm^2 of

foliage per day (Ferro et al., 1985). To ensure viability of the potato industry, farmers can prevent the spread of the potato beetle between crops through crop rotation, crop scouting and altering planting times. Despite their efforts, the use of pesticides is often needed to control the potato beetle population. The Colorado potato beetle continues to cause problems because it develops pesticide resistance at an extremely rapid rate; the beetle is now resistant to 30 different active ingredients (Forgash, 1985; Mota-Sanchez et al., 2006).

1.9 Persistent organic pollutants

Organochlorines such as DDT, heptachlor, dieldrin and endosulfan, have historically been popular agricultural pesticides. These chemicals are very stable in the environment and are classified as persistent organic pollutants (POP) under the United Nations Environment Program (UNEP) Stockholm Convention of 2001 (UNEP Stockholm Convention, 2001). Chemicals are considered to be POP if they meet four criteria: 1) persist in the environment, 2) show high affinity for bioaccumulation, 3) capable of long distance transport and 4) show negative effects on humans or the environment (UNEP Stockholm Convention, 2011). Although many organochlorines have now been banned or regulated around the world, endosulfan continues to be used in some countries. In May 2011, the UNEP acknowledged the risks of endosulfan and it was added to the list of POP (UNEP Stockholm Convention, 2011). The World Health Organization (WHO) has also added endosulfan to Annex III of the Rotterdam Convention in 2011, whose purpose is to provide information on chemicals that have been restricted or banned by two or more parties bound by the convention (WHO, 2011).

1.10 Endosulfan

The global use of endosulfan through 2000 was estimated to be 338 kilotons, with India being the largest consumer (Li and Macdonald, 2005). In 2002-2003 the sale of endosulfan in Canada was estimated to be 21,000 kg, with over half sold in Atlantic Canada (Brun et al., 2008). Until recently, endosulfan was sanctioned for use in Canada with guidelines and restrictions. The United States Environmental Protection Agency (US EPA) announced in 2010 that it would be implementing a phase out program of endosulfan use (US EPA and Office of Pesticide Programs, 2010). The Pest Management Regulatory Agency (PMRA), Canada's governing body of pesticides, have also initiated an endosulfan phase out plan by 2016 (Health Canada, 2011). The production of endosulfan in Canada will cease by the end of 2014 and the sale and use will be terminated by the end of 2015 and 2016, respectively (Health Canada, 2011). Despite the phase out of endosulfan in Canada, PMRA has a list of guidelines that must be followed until 2016. A mandatory 10 m vegetative buffer is required around the perimeter of an exposed area if it is near a marine or freshwater environment, in addition to an overall 30 m buffer zone from a marine or freshwater source (CCME, 2010). PMRA has also created an endosulfan user guide that contains information on the application of the pesticide, how many applications per year and the minimum number of days between treatments (Health Canada, 2011).

Endosulfan is a popular pesticide that is used on corn, beans, potatoes and other agricultural fields to combat insect pests and was first introduced to Canada in 1956 (CCME, 2010). Endosulfan is an organochlorine in the family of chlorinated hydrocarbons, denoted by the high number of chlorines in the chemical structure.

Commercial endosulfan is composed of α-endosulfan and β-endosulfan in a 7:3 ratio and is a γ-aminobutyric acid (GABA) chloride channel receptor inhibitor in nerve tissue (Bloomquist, 2003; Weber et al., 2010). In the absence of endosulfan, a neurotransmitter would bind to GABA chloride channel receptors and cause the nerve cell to hyperpolarize, preventing multiple action potentials. Endosulfan acts as an inhibitor preventing chloride channels from opening resulting in hyperexcitation, convulsions and potentially death of the organism (Bloomquist, 2003).

Environmental toxicology studies on endosulfan attempt to identify sub-lethal effects and their mode of action. Sub-lethal effects are defined as changes in biochemistry, physiological process, growth, development, reproduction, behaviour and damage to cells, tissues and organs (Newman, 1998). Endosuflan causes changes in immune cell function (Tellez-Bañuelos et al., 2009), changes in histology and physiology, activation of oxidative stress (Dorts et al., 2009; Dorval et al., 2003; Ezemonye and Tongo, 2010; Sharma et al., 2011) and acts as an endocrine disrupting chemical (Palma et al., 2009; Sharma et al., 2011; Zou and Fingerman, 1997).

The presence and persistence of endosulfan in the environment is disconcerting because it can migrate between different domains (air, water and soil) in the environment and possess severe ecotoxicological risks to non-target organisms (Weber et al., 2010). The fate of endosulfan is highly dependent on where it is found in the environment and environmental conditions.

1.11 Endosulfan in water

Although endosulfan is an agricultural pesticide; its presence has been detected in freshwater and marine environments from runoff and atmospheric deposition. Over the past 50 years, there have been 51 reported fish kills on PEI, among which 10 were suspected to involve endosulfan (PEI Department of Agriculture and Forestry, 2012). The goal of implementing mandatory 10 m vegetative buffer and overall 30 m buffer zones was to reduce the pesticide load in soil that had the potential to runoff during heavy rain events (Dunn et al., 2010). Soil on PEI is susceptible to erosion and runoff due to its sandy nature, low organic content, poor structure and high levels of rain (PEI Department of Agriculture and Forestry, 2003). Dunn et al. (2010) showed that although the concentration of pesticide was reduced by buffer zones, the remaining concentration can still be deleterious to sensitive species such as the water flea (Daphnia magna). Preventative measures including crop rotation, soil management and increased vegetative buffers to 15 m have reduced the number of annual fish kills on PEI; however contaminants continue to enter aquatic environments (PEI Department of Agriculture and Forestry, 2012). Water quality monitoring of Canadian aquatic ecosystems has detected endosulfan at test sites throughout Atlantic Canada during scheduled sample collections (Environment Canada, 2011). However the greater concern is levels after heavy rain events. Runoff analysis from farms on PEI found that the concentration of α-endosulfan and β-endosulfan were reduce by 98 and 97% respectively with a 10 m vegetative buffer and 100% with a 30 m buffer (Dunn et al., 2010). Despite the percent reduction in the concentration by the buffer zones, the measured concentration of α-endosulfan and β-endosulfan were 0.19 and 0.31 µg/L for the 10 m buffer zone and 0.07 and 0.06 µg/L for the 30 m buffer zone (Dunn et al., 2010). The measured

concentrations after the 10 m buffer are higher than the current CCME short term exposure limit for the marine water guideline for the protection of aquatic life of 0.09 µg/L (CCME, 2010).

Occurrences of pesticide runoff are a concern in other agricultural regions in Canada such as the Okanogan Basin in British Columbia. Both the endosulfan parent isomers, α-endosulfan and β-endosulfan, and endosulfan sulfate were detected in runoff water in the Okanogan Basin between 2003-2005 with the metabolite endosulfan sulfate detected at the highest concentration of 0.109 µg/L (Environment Canada, 2011). As populations of amphibians are declining throughout the world, a concern in the Okanogan Basin is that endosulfan, in conjunction with other agricultural pesticides, could be affecting the local Pacific treefrog (Pseudacris regilla) and great basin spadefoot toad (Spea intermontana) (De Jong Westman et al., 2010).

Endosulfan has low water solubility of 0.32 mg/L (CCME, 2010). Despite its low solubility, the aquatic half-life of α-endosulfan is 23-27 h and β-endosulfan is 22-27 h; however these half-lives change depending on the initial concentration, water temperature, pH and oxygen level (Guerin, 2001; Weber et al., 2010). Endosulfan is degraded into several metabolites, the most common being endosulfan sulfate. The US EPA considers endosulfan sulfate a chemical of concern since it can elicit similar effects as its parent isomers and is more persistent than both α-endosulfan and β-endosulfan (US EPA Office of Chemical Safety and Pollution Prevention, 2010).

1.12 Endosulfan in sediment

Chemicals are considered to be POPs if they have a log octanol-water partition coefficient (log K_{ow}) > 5, signifying that they partition easily into sediment (Weber et al., 2010). Technical grade endosulfan has a log K_{ow} of 3.55, however α-endosulfan and β-endosulfan have log K_{ow} near 5, 4.94 and 4.78 respectively. The increased partition of endosulfan and its parent isomers into sediment is thought to prolong their presence in marine and aquatic environments (Weber et al., 2010). The increased stability of endosulfan in sediment is apparent in the longer half-lives of the isomers, 7-75 days for α-endosulfan and 33-376 days for β-endosulfan (Weber et al., 2010). A concentration of 50 µg/kg endosulfan in sediment caused decreased colonization and overall growth of the polychaete species Streblospio benedicti; however it did not significantly affect copepods Pseudobradya pulchella in the same study (Chandler and Scott, 1991). Hose et al. (2002) found similar results where endosulfan did not affect overall benthic macroinvertebrate populations. In sediment, the breakdown of endosulfan can occur through hydrolysis and photolysis, and degradation by the presence of microorganisms. Successful bioremediation requires ideal microbial conditions including, soil pH, moisture content, carbon source, an anaerobic environment, non-lethal concentration of endosulfan to the microorganism community, and an appropriate size of microorganism inoculum (Awasthi et al., 2000; Guerin, 1999; Siddique et al., 2003). Despite the ability of endosulfan to be degraded through biotic and abiotic factors, it continues to be a POP.

1.13 Endosulfan in air

Endosulfan enters the atmosphere through spray drift and post spray volatilization, and is susceptible to atmospheric long range transport (Weber et al., 2010; White et al., 2006). A global atmospheric passive sampling (GAPS) initiative has detected endosulfan in all areas of the world and in regions such as the Arctic, where the pesticide has never been used (Weber et al., 2010). Environment Canada performed a similar study in 1998-1999 of pesticide concentrations in air throughout PEI at both agricultural and non-agricultural sites. Endosulfan was detected on agricultural sites that had used the pesticide, on farms that had not used the pesticide and at non-agricultural sites (White et al., 2006). Atmospheric monitoring of other agricultural regions in Canada between 2004-2005 determined that endosulfan was the most commonly detected organochlorine across the country (Yao et al., 2008). The presence of endosulfan in the atmosphere is yet another potential source of contamination of marine and freshwater environments.

1.14 Endosulfan ingestion

Most of research on the effects of endosulfan has been through external exposure (water, sediment and air) and there has been limited research into ingestion as an exposure route. The spoonfinger rubble crab larvae (Leptodius floridanus) fed brine shrimp (Artemia salina) contaminated with another organochlorine, dieldrin, showed considerably less toxicity than water exposure (Epifanio, 1972). Neonates from the water flea (D. magna) fed endosulfan contaminated phytoplankton species (Pseudokirchneriella subcapitatum) had little endosulfan bioaccumulation (DeLorenzo

et al., 2002). Despite the limited response in both experiments, toxic effects can also be life stage specific. Larvae exposed to dieldrin contaminated food did not ingest enough food to cause an effect, whereas D. magna neonates were considered to have a lower lipid content decreasing the ability to bioaccumulate (DeLorenzo et al., 2002; Epifanio, 1972). Interestingly, a study of the bioaccumulation of PCBs and chlorinated pesticides in seals, fishes and invertebrates from the White Sea in Russia detected chlorinated contaminants at all trophic levels (Muir et al., 2003). Although the toxicity of endosulfan through ingestion is not fully understood, it should be considered a viable route of exposure.

1.15 Effects of endosulfan on survival

Environmental toxicity can be described using the concept of lethal concentration that kills (LC_{50}) or results in negative effects (EC_{50}) in 50% of the test population during a specific period. Comparing the toxicity of a substance among species can be difficult depending on duration and end point of the test (LC_{50} or EC_{50}). Freshwater fish are variably sensitive to acute endosulfan exposure with 96 h LC_{50} concentrations ranging from 0.1 µg/L in the common carp (Cyprinus carpio) to 5780 µg/L in the spotted snakehead (Channa punctata) (CCME, 2010). Invertebrate species can be sensitive to certain toxicants and therefore the EC_{50} value may be reported as opposed to the LC_{50} value. The 48 h LC_{50} value for endosulfan in water flea (D. magna) was 1300 µg/L compared to the 48 h EC_{50} value of 400 µg/L that caused immobility or death in affected animals (Environment Canada, 2012 unpublished). Amphibians, specifically tadpoles, are becoming an increasingly popular test species. The 96 h acute

LC_{50} of endosulfan exposure to the common toad (Bufo bufo) was 430 µg/L (Brunelli et al., 2009). Endosulfan exposure experiments are predominantly performed on freshwater species; however research into the effects of endosulfan exposure on marine and estuarine species is increasing. Schimmel et al. (1977) measured acute toxicity of endosulfan in 5 estuarine species: pink shrimp (Penaeus duorarum), grass shrimp (Palaemonetes pugio), pinfish (Lagodon rhomboides), spot croaker (Leiostomus xanthurus) and striped mullet (Mugil cephalus) and reported 96 h LC_{50} values of 0.04, 1.3, 0.3, 0.09 and 0.38 µg/L respectively.

1.16 Effects of endosulfan on growth and development

Both acute toxicity and chronic exposures to low levels of endosulfan can have serious long term effects on growth and development in marine and freshwater species. Moulting is a crucial developmental process that allows arthropods to grow and increase in size. Endosulfan has repeatedly been identified as an endocrine disruptor and moult inhibitor resulting in developmental delays (Meng and Zou, 2009; Montagna and Collins, 2007; Palma et al., 2008; Zou and Fingerman, 1997, 1999; Zou, 2005). D. magna exposed to endosulfan concentrations of 0.10 and 0.15 mg/L resulted in an increase in the number of days required to undergo the moulting process (Zou & Fingerman, 1997). Freshwater prawns (Palaemonetes argentinus) exposed to sublethal concentrations of endosulfan also exhibited deviations from the normal moult cycle (Montagna and Collins, 2007). Although endosulfan negatively affects the moult cycle, the mode of action to produce such results is not well understood (Zou, 2005). Endosulfan could act as an antagonist to the ecdysone receptor (EcR) preventing

downstream regulation of target genes (Meng and Zou, 2009; Zou and Bonvillain, 2004; Zou and Fingerman, 1999). The metabolite endosulfan sulphate also causes significant delays in moulting in D. magna (Palma et al., 2009). Changes in the moult cycle can have dramatic effects on many different aspects of the life cycle such as delay in metamorphosis, reduction in overall size and lowered reproduction (Capuzzo et al., 1984; Palma et al., 2008; Sharma et al., 2011). Endosulfan exposure of the common toad (B. bufo), great basin spadefoot frog (S. intermontana) and Pacific tree frog (P. regilla) has caused delayed development through metamorphosis, morphological abnormalities and behavioural changes (Brunelli et al., 2009; De Jong Westman et al., 2010).

1.17 Effects of endosulfan on immunity and oxidative stress

Research into the effects of pesticides on immune response in insects and crustaceans is limited compared to the study of mammalian species (James and Xu, 2011). Endosulfan negatively impacts the immune system of invertebrates. Unlike vertebrates, invertebrates do not have an adaptive immune system and rely solely on innate immune responses such as phagocytosis, melanization and increases in total hemocyte number (Cerenius and Söderhäll, 2004; Chang et al., 2006; Galloway and Depledge, 2001; Johansson and Söderhäll, 1989; Söderhäll and Cerenius, 1998). Immunological responses vary depending on the stressor and species, for example when four corixidae (water boatman) species were exposed to endosulfan they exhibited different levels of phenoloxidase activity (Trekels et al., 2011). A major detoxifying organ in crustaceans is the hepatopancreas and 17 genes were identified as up/down

regulated after methoprene exposure in the lobster (Horst et al., 2007). Changes in gene expression in the hepatopancreas could indicate signs of stress and immune response (Horst et al., 2007).

Exposure to a toxicant activates the detoxification pathway consisting of phase I and phase II enzymes. Phase I metabolizing enzymes are from the cytochrome p450 family (CYP) and are the first line of defence in the elimination of toxicants (Xu et al., 2005). The exposure of juvenile lobster (H. americanus) to chlorinated pesticides resulted in an increase in the expression of CYP450 in the hepatopancreas (Snyder and Mulder, 2001) and endosulfan exposure to Drosophila melanogaster elicited an up regulation of 4 different CYP enzymes (Sharma et al., 2011). After the toxicant has entered the organisms, it can bind to a series of nuclear receptors that act as transcription factors and regulate target genes responsible for phase I and II metabolism (Xu et al., 2005). Examples of phase II enzymes that have been monitored for the induction of stress response are glutathione S-transferase (GST), uridine diphospho glucuronsoysltransferase (UGT) and sulfotransferase (SULT). Field crabs (Paratelphusa hydrodromus) exposed to sublethal concentrations of endosulfan had increased expression of GST in the hepatopancreas after only 48 h and GST levels remained significantly higher than control animals throughout an 8 day endosulfan exposure experiment (Yadwad, 1989). Another parameter that can be monitored to identify stress is the presence of heat shock proteins (HSP) which prevent protein denaturation and assist in protein folding (Chang et al., 1999; Dorts et al., 2009). Specific HSP may vary depending on the type of stress and species. However endosulfan has activated hsp22& hsp23 in D. melanogaster (Sharma et al., 2011), hsp70

in lobster (H. americanus) (Snyder and Mulder, 2001) and hsp70 and hsp90 in black tiger shrimp (Penaeus monodon) (Dorts et al., 2009).

Endosulfan has toxicological effects on various marine and freshwater species. B. bufo tadpoles exposed to environmentally relevant concentrations of endosulfan showed changes in behaviour, deformities and delays in metamorphic development (Brunelli et al., 2009). Endosulfan is considered to be an EDC, and although it does not appear to have estrogenic effects (Palma et al., 2009), it has caused significant delays in the moult cycle in D. magna (Palma et al., 2009; Zou and Fingerman, 1997), D. melanogaster (Sharma et al., 2011) and P. argentines (Montagna and Collins, 2007). Microarray results of D. melanogaster exposed to endosulfan elicited changes in expression of genes involved in development, immune and oxidative stress and metabolism (Sharma et al., 2011).

1.18 Microarray analysis of gene expression

Microarrays are high throughput analytical devices that allow the simultaneous monitoring of thousands of gene expression events within an organism (Schena, 2003). Small cDNA or oligonucleotide target sequences are attached to a glass slide and hybridize to a labelled single stranded probe. Microarrays provide quantitative analysis for both abundant and rare transcripts (Schena, 2003).

Since their development in the early 1990s, microarrays have become a valuable resource for studying genome wide gene expression in different species and across many disciplines (Yauk and Berndt, 2007). Microarrays are useful as diagnostic and screening tools in the medical field (Bilban et al., 2002) and as species genomes continue to be

sequenced, the uses of microarrays are also evolving. Microarray technology is now being applied to toxicological and ecotoxicological studies to monitor the effects of environmental contaminants (Lettieri, 2006). Traditional toxicology endpoints include survival, changes in behaviour, morphology and immune response; microarrays allow for a complete overview of how toxicants elicit a response at the genomic level and are useful in identifying what pathways might be affected by toxicant exposures (Ju et al., 2007).

With the increasing use of microarrays, it is necessary to create a standard reporting method to allow for experimental reproducibility and data mining. Although the proposed reporting method is not mandatory for publication, it is becoming highly recommended to follow the guideline known as the Minimum Information About Microarray Experiments (MIAME) that requires description of the following: 1) experimental design, 2) array design, 3) sample, 4) hybridization, 5) experimental measurements, and 6) normalization controls (Brazma et al., 2001).

Over the years several techniques including expressed sequence tag (EST), subtractive cloning, differential display, serial analysis of gene expression (SAGE), and microfluid dynamic array have been used for monitoring gene expression (Bustin et al., 2009). The method chosen for gene expression analysis dependends on the scientific question and level of throughput required for the experiment. A combination of techniques can be used as was done in this study utilizing EST sequencing, microarrays and reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Microarrays now exist for many aquatic species including the sheephead minnow (Cyprinodon variegatus), Atlantic salmon (Salmo salar), common carp (C. carpio), rainbow trout (Onchohynchus mykiss) and zebrafish (Danio rerio) and are important

tools for toxicogenomic studies (Ju et al., 2007). Organisms can be exposed to an estimated 70,000 commercially available products; this has resulted in the focus of current toxicogenomic research to identify unique expression profiles for a specific toxicant or chemical family and identify modes of action (Neumann and Galvez, 2002; Soetaert et al., 2007). Gene expression changes have been analyzed prior to development of microarrays. Expression levels are dependent on many factors and identifying how a toxicant affected expression levels was difficult. With the ability to monitor gene expression profiles and gene interactions, greater understanding of the effects of toxicant exposure can be achieved.

1.19 Hypothesis and objectives

Although precautionary measures are in place to prevent pesticide spray drift and agricultural runoff, elevated concentrations of endosulfan are being detected in aquatic environments and threaten the lucrative lobster industry. The purpose of this study is to determine the effects of the organochlorine pesticide endosulfan on a non-target organism, the American lobster Homarus americanus. It is hypothesized that exposure of H. americanus larvae to endosulfan will cause a delay in the moult cycle preventing the stage III larvae from undergoing metamorphosis to become stage IV postlarvae. In addition, it is hypothesized that the severity of the developmental delays will intensify as the endosulfan exposure concentrations increase and that there will be significant changes in gene expression levels in animals that survived the exposure experiment. The main objectives of this research are to:

- Determine the effects of endosulfan on survival and metamorphosis in H. americanus.

- Identify genes that are significantly differentiated in various endosulfan concentrations.

- Elucidate what pathways and biological processes might be affected by endosulfan exposure.

- Identify potential biomarkers for endosulfan exposure.

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Chapter 2: ANALYSIS OF GENE EXPRESSION IN HOMARUS AMERICANUS LARVAE EXPOSED TO SUBLETHAL CONCENTRATIONS OF ENDOSULFAN DURING METAMORPHOSIS

2.1 Introduction

Two major industries in Atlantic Canada and Québec are agriculture and fisheries. Although they may appear as two independent entities, there is a direct link between responsible agricultural practices and the quality of neighbouring marine and freshwater environments (Schafer et al., 2007). Agricultural runoff after rain events increases the risk of pesticides and other agricultural products contaminating local water ways and inshore marine habitats. Although an increasing number of environmental contaminants enter into aquatic habitats, the long term effects on non-target organisms are unknown. Anthropogenic stressors, including the presence of pesticides, impact populations of numerous species and contribute to changes in community structure within rivers and streams (De Jong Westman et al., 2010; Schafer et al., 2007).

Agriculture and fishing industries must be able to co-exist where the practices of one industry do not harm the other. Contamination of marine and freshwater environments with pesticides can occur through spray drift, atmospheric transport, precipitation deposition, waste water and contaminated sediment runoff (Nowell et al., 1999). Consequently, impacts on different species or different life stages may vary both spatially (geographic location) and temporally (season). The American lobster, H. americanus, is one of the last viable commercial fisheries in Atlantic Canada. Lobster larval stages are pelagic during their early development and are increasingly susceptible to pesticides from agricultural runoff and spray drift. Suspended contaminated soil particles eventually settle to the substrate and have the potential to negatively affect

postlarval and mature lobster. Sustaining healthy lobster populations is heavily dependent on recruitment and survival of lobster larvae (Fogarty, 1995) and understanding the effects of environmental contaminants during larval stages is important.

Ecotoxicology is the study of pollutants and their effects on populations and communities within an ecosystem (Kappeler, 1979). As technology advances, the methods and assays available to study the effects of pollutants at different levels of biological organization are also changing. Ecotoxicogenomics combines the fields of ecology, toxicology and genomics in the study of pollutants (Iguchi et al., 2007). Specifically, the application of high throughput microarrays is becoming a popular research tool in ecotoxicological studies to simultaneously monitor large numbers of genomic changes that occur in an organism. As more genomes become sequenced and fully annotated, microarray experiments will provide systematic information on how pollutants impact the normal functioning of an organism. There is already ongoing research into using gene sets to predict and identify toxicological substances in mice (Thomas et al., 2001), however there have been no such comparison and identification studies done on aquatic species.

Toxicological databases are being created that compile information on the proteomic, metabolomic, genomic and traditional toxicology data in an attempt to link related information and predict the potential effects of toxicants (Miracle and Ankley, 2005). Although the genome of the American lobster is not fully sequenced, a custom microarray has been created based on unique expression sequence tags (ESTs) providing the opportunity to study the ecotoxicogenomic effects of contaminants on developing lobster larvae (Greenwood and Towle, unpublished).

The organochlorine pesticide endosulfan is a persistent organic pollutant (POP) and is easily transported between environments (air, water, sediment, soil) and geographical locations (Weber et al., 2010). In 2002, 4,500 kg of endosulfan active ingredient (a.i.) was sold on Prince Edward Island (Health Canada, 2009) and despite recent implementation of a phase out program in Canada, endosulfan use is permitted on potato crops until December 31, 2016 (Health Canada, 2011). Agricultural runoff causing fish kills has been a problem on PEI since the 1960s. Endosulfan has been implicated as a probable cause in multiple fish kills throughout the Island since 1975 (PEI Department of Agriculture and Forestry, 2012). Endosulfan has moult inhibiting effects on other crustaceans (Palma et al., 2009; Sharma et al., 2011; Zou and Fingerman, 1997) and developmental effects on numerous other species (Brunelli et al., 2009; Gormley and Teather, 2003; Han et al., 2011; Rohr et al., 2003). However its effects at the genomic level are largely unknown.

In the present study, lobster larvae were exposed to environmentally relevant concentrations of endosulfan during metamorphosis to determine the effects on survival, and development while identifying differentially expressed genes through microarray analysis. Biologically interesting genes were further selected for RT-qPCR validation of the microarray data. The results of this study are the first to associate the effects of endosulfan on development at a genomic level during metamorphosis in H. americanus.

2.2 Materials and methods

2.2.1 Collection and holding of lobster larvae for testing

American lobster (H. americanus) larvae were collected from the Coastal Zone Research Institute-Homarus Inc. in Shippagan New Brunswick (NB) on June 2, 2010 (Appendix A). Approximately 3000 stage I lobster larvae were collected from the hatchery on the day of hatching and transported to the Environment Canada Toxicology Laboratory in Moncton, NB. During transportation, larvae were held in clean 10 L pails in aerated natural sea water. Water temperature and aeration were monitored throughout transportation and adjusted as needed. After arrival at the laboratory, water quality testing was performed on the transportation water and the culture holding tank water. The holding tank had a temperature of 20 ºC ± 2 °C, salinity of 30 ± 2 ‰, dissolved oxygen (DO) 90-100% saturation and pH 7.50-8.50. Ammonia levels were monitored using an ammonia alert indicator (safe= < 0.02 mg/L, alert= 0.05 mg/L, alarm= 0.2 mg/L, toxic= 0.5 mg/L) (Seachem, Madison, GA).

Lobster larvae were held in highly aerated natural sea water to reduce the occurrence of cannibalism within the holding tank. Culture water was renewed a minimum of 50% daily based on ammonia level and overall cleanliness of the water. The holding tank water was renewed by siphoning into a 10 L perforated bucket to prevent animals from escaping, yet allowing old food and dead animals to be removed. Temperatures, DO, pH, salinity were measured and an alert indicator was used to monitor for unacceptable ammonia levels daily.

Currently no commercially available diet exists for lobster larvae. Feedings were based on research that indicates high lipid and protein content are required for optimal

growth and survival of larvae (Conklin, 1995; D'Abramo and Conklin, 1985; Fiore and Tlusty, 2005). Larvae were fed twice daily (morning and afternoon) with 2 g of dry fish flakes (Salt Creek, Salt Lake City, UT), 2 g of live brine shrimp (Artemia sp.) (Salk Creek, Salt Lake City, UT) and 15 g of frozen brine shrimp daily (Hikari, Hayward, CA) per 2000 larvae (tank biomass ~20 g). Lobster larvae were cultured until the majority reached stage III (8 days after acquisition) when the endosulfan exposure experiment was started.

2.2.2 Preparation of stock solutions of endosulfan

The stock solution A was prepared by adding 0.25 g of Thiodan® 50 WP (50% endosulfan) to a 1 L glass volumetric flask filled with distilled water to give a final concentration of 125 µg/ml of endosulfan. Stock B was prepared by adding 10 ml of Stock A to 100 µl of distilled water resulting in a final concentration of 12.5 µg/ml of endosulfan.

2.2.3 Chronic endosulfan exposure

Endosulfan solutions were prepared in 35 L glass aquarium filled with natural sea water and the addition of the corresponding volume of Stock B solution (Table 2.1). Test solutions were poured into well labelled 1 L mason jars (30 replicates/ concentration) and were placed into a temperature regulated water bath at 20 ± 2 ºC. Glass tipped airlines were added to each jar and the test solutions were acclimatized to 20 ºC and DO of 90-100% saturation prior to the addition of the stage III larvae. Temperature, DO, pH and salinity were recorded for each test concentration. Test animals were removed from the holding tank, collected individually in small plastic medicine cups and added to the mason

jars. Water quality parameters were maintained within the following: temperature 20 ± 2 ºC (18.1-21.5 °C), DO 90-100% saturation (7.4-7.8 mg/L), pH 7.50-8.50 (7.53-7.85) and salinity 28±2 ‰ (28 ‰). Each larva was fed 5 mg of dry fish flakes, 5 mg of frozen brine shrimp and 100 live Artemia daily. Water quality was monitored daily for 14 days and observations on survival and moulting were recorded. The test was renewed with freshly made stock solutions three times per week. Water samples were collected in 4 L glass amber jugs for chemical analysis at the beginning, middle (day 8) and near the end (day 12) of the exposure on both fresh and aged solutions (Table 2.2).

Table 2.1: Required volumes of the 12.5 µg/L Stock B endosulfan solution to achieve nominal test concentrations of 1.0, 0.3, 0.1, 0.03, 0.01 and 0 µg/L respectively.
Exposure solutions were made fresh three times per week until termination of the test on day 14.

Endosulfan Test Concentration (µg/L)	ml of Stock B in 35 L natural seawater
1.0	2.8
0.3	0.84
0.1	0.280
0.03	0.084
0.01	0.028
Control	0

Table 2.2: Endosulfan exposure solutions collected for chemistry analysis. Fresh and aged solutions were collected at the beginning, middle and near the end of the exposure experiment.

Date (2010)	Renewed	Fresh Solution Collected	Aged Solution Collected
9 June	Started	Yes	No
16 June	Yes	Yes	Yes
21 June	Yes	Yes	Yes
23 June	Terminated	No	No

2.2.4 Water sample chemical analysis

Water chemical analysis was performed in the organic chemistry section of the Environment Canada Atlantic Laboratory for Environmental Testing (ALET) in Moncton NB. Briefly, endosulfan exposure samples were extracted into dichloromethane (DCM) using a liquid/liquid extraction method. As quality control, known surrogates (γ-chlordene, PCB 103 and PCB 198) were added prior to extraction and an internal standard (Dichlorobiphenyl) was added prior to measurements to ensure the extraction and detection procedures were operating correctly. Blank seawater samples and a spike solution with a known endosulfan spike-in concentrations were also extracted for further quality control. Gel permeation chromatography (GPC) and silica column filtration was performed on the extracted samples. A final extract volume of 0.5 ml was analyzed using gas liquid chromatography with a dual column electron capture detector (GC-ECD) allowing for quantification and verification. Endosulfan metabolites α-endosulfan, β-endosulfan and endosulfan sulfate were all measured.

2.2.5 RNA extraction

At the conclusion of the exposure, remaining animals were measured, weighed and preserved in 1.5 ml tubes containing TRIzol® (Invitrogen, Burlington, ON). The tubes were placed in the FastPrep®-24 (MP Biomedicals LLC, Solon, OH) and homogenized during 3 × 20 s pulses at 4 m/s. Samples were transferred to cryovials, flash frozen in liquid nitrogen and stored at -80 ºC.

RNA was extracted using phenol-chloroform and the Qiagen RNeasy® Mini Kit (Qiagen, Germantown, MD) as per a modified version of the manufacturer's instructions (Appendix B). Briefly, after preserved samples were thawed, 200 µl of chloroform was pipetted into a 1.5 ml RNase free tube containing the homogenate material and inverted 15 times. Samples were incubated at room temperature for 3 min before they were centrifuged at 8000 x g for 15 min at 4 ºC using the Eppendorf 5415R centrifuge. The supernatant was carefully transferred into a new 1.5 ml RNase free tube ensuring that no debris from the protein layer was accidentally transferred. An equal amount of 70% ethanol (EtOH) was added to the supernatant and gently mixed by pitetting up and down. Seven hundred microlitres of supernatant were filtered through an RNeasy® filter column with a combination of RW1 and RPE buffer solution washes. Two 30 µl elutions were performed using nuclease free water by centrifugation at 8000 x g for 2 min each, for a total of 60 µl of RNA extract. Two aliquots of 1:10 dilution of samples RNA were prepared with nuclease free water for quantity and quality verification. Two microlitres of RNase inhibitor were added to the remaining 56 µl of eluted RNA and samples were stored at -80 ºC for subsequent fluorescent dye labelling.

2.2.6 Reference sample collection and pooling

The microarray reference sample was composed of 15 larvae from both stage III and stage IV for a total of 30 animals. Individuals were placed into 1.5 ml tubes containing 1 ml of TRIzol® (Invitrogen, Burlington, ON), loaded into FastPrep® -24 (MP Biomedicals LLC, Solon, OH) and homogenized as above (2.2.5). The concentration of RNA extracted was measured and volumes containing 1330 ng of material were pooled to ensure equal

representation in the reference sample. The reference sample was stored in 4 µl aliquots with an approximate concentration of 266 ng/µl at -80 ºC.

2.2.7 Assessing RNA quantity

RNA quantity was assessed using the Thermo Scientific NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Briefly, 2 µl of nuclease free water was pipetted into the wells of the NanoDrop to initialize and blank the machine after which RNA samples were analyzed with the RNA-40 option. Samples with a concentration greater than 200 ng/µl and having a 260/280 absorbance ratio of 2 were considered suitable for microarray use.

2.2.8 Assessing RNA quality

The quality of RNA was assessed using the Bio-Rad Experion™ (Bio-Rad, Hercules, CA) automated electrophoresis system. One microlitre of RNA was added to a 0.2 ml tube containing 6 µl of gel loading buffer and denatured at 70 ºC for 2 min. The LabChip® was primed with 9 µl gel stain solution and 9 µl pure gel was added to the designated well. Seven microlitres of sample were added to wells 1-12 and compared against a standard ladder. Samples with two distinct bands (18S & 28S rRNA) and an RNA quality indicator (RQI) of >7 were selected for labelling.

2.2.9 Amplification and labelling of RNA using Agilent Low Input Quick Amp Kit

The Agilent Low Input Quick Amp Kit -two colour (Agilent, Mississauga, ON) required an initial concentration of 25-200 ng of RNA in 1.5 µl of solution to be added to 2 µl of nuclease free water in place of the Agilent spike in control as the lobster microarray slides were custom made. A 1.8 µl volume of T7 promoter primer mix was added to each sample (Table 2.3) and denatured in the Eppendorf Mastercycler® (Eppendorf, Mississauga, ON) at 65 ºC for 10 min followed by incubation on ice for 5 min. Immediately prior to use, the cDNA Master Mix was prepared and 4.7 µl was added to each sample (Table 2.3). Samples were mixed well and incubated at 40 ºC for 2 h, 70 ºC for 15 min, followed by 5 min on ice. To ensure all enzyme activity had ceased, samples were placed in the -80 ºC freezer overnight. The following day, immediately prior to use, two Transcription Master Mixes were prepared; endosulfan exposure samples were labelled with a Cy3 fluorescent dye and the reference samples were labelled with a Cy5 fluorescent dye. Six microlitres of the appropriate master mix was added to each sample (Table 2.3), incubated at 40 ºC for 2 h and stored at -80 ºC.

Table 2.3: The components of the Agilent Low Input Quick Amp Kit master mixes and the required volume per sample. Master mix calculations were based on n+1 to ensure there was enough solution for each sample.

Master Mix	Volume (µl)
T7 Promoter Primer Mix	**1.8**
T7 Promoter Primer	0.8
Nuclease Free Water	1.0
cDNA Master Mix	**4.7**
5X First Strand Buffer (pre-warmed)	2.0
0.1M DTT	1.0
10mM dNTP	0.5
AffinityScript RNase Block Mix	1.2
Transcription Master Mix	**6.0**
Nuclease Free Water	0.75
5X Transcription Buffer	3.2
0.1M DTT	0.6
NTP mix	1.0
T7 RNA Polymerase Blend	0.21
Cyanine 3 or Cyanine 5	0.24

2.2.10 Purification of amplified and labelled RNA

A Qiagen RNeasy® Mini Kit (Qiagen, Germantown, MD) was used to purify amplified and labelled RNA. Briefly, 84 µl of nuclease free water was added to each sample to bring the final volume to 100 µl. Three hundred and fifty microlitres of RLT buffer and 250 µl of 100% ethanol were added and mixed thoroughly by inverting 15 times. All 700 µl of the sample was pipetted onto an RNeasy® filter column, centrifuged at 8000 x g at 4 ºC for 30 s and the waste discarded. The RNeasy® column was transferred to a new collection tube and a series of washes with RPE buffer was performed. Samples were eluted into 30 µl of nuclease free water by centrifugation at 8000 x g at 4 ºC for 30 s. The NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to determine the concentration and dye incorporation. Samples with a concentration of 200 ng/µl and dye incorporation of 2.0 pmol/µl were considered acceptable for microarray hybridization and stored at -80 ºC.

2.2.11 RNA fragmentation

Both endosulfan and reference RNA samples were fragmented with Ambion RNA fragmentation reagents (Ambion, Burlington, ON) prior to hybridization. Briefly, 1800 µg of labelled RNA was added to nuclease free water for a total volume of 9 µl. One microlitre of 10X fragmentation buffer was added to each sample and incubated at 70 ºC for 15 min. To terminate the reaction, 1 µl of stop solution was added and samples were placed on ice until hybridization injection.

2.2.12 Microarray Platform

There are no commercially available microarray slides for the American lobster (Homarus americanus); therefore a microarray platform was made from determining the nucleotide sequence of 20,000 ESTs from a lobster cDNA library (Greenwood & Towle, unpublished). Samples were taken from 10 different tissues from 2 male and 2 female intermoult and postmoult lobsters. RNA was extracted and an equal amount of RNA from each tissue (175 μg/each) was used to create a pooled sample. A total of 29,636 lobster expressed sequence tags (EST) were available in GenBank at time of microarray construction. Through 2 different clustering methods, CLOBB and TIGR, 15,864 unique sequences were identified. The resulting ESTs were functionally annotated using BLAST2GO (Conesa et al., 2005) and BLASTx. Among the top10 BLASTx hits, the most descriptive or likely protein was selected manually as the "annotation of best fit". The 14,592 EST identified to be used on the microarray were designed using Array Designer 4 software and synthesized by Integrated DNA Technologies (IDT) (IDT, Coralville, IA). A total of 15,376 50-mer oligonucleotides (14,592 EST, 210 Sigma Alien controls, 78 buffer controls, 80 GFP landmark controls and 416 buffer controls) were submitted to the Vancouver Prostate Centre DNA Microarray facility, Vancouver British Columbia, for printing on aminosaline glass slides. Each slide contained two (dual) arrays, resulting in each of 15,376 oligonucleotides being printed in duplicate using the same array template.

2.2.13 Pre-hybridization and hybridization

The Tecan HS 4800 Pro (Tecan, San Jose, CA) was pre-hybridized according to manufacturer's instructions (Appendix F). Once primed, the hybridization mixture containing 825 ng of both Cy3 and Cy5 labelled material, 50 µl of Ambion Hyb solution #3 (Ambion, Burlington, ON) and 1 µl of LNA dt blocker (Genisphere, Hatfield, PA) was injected into the microarray chamber injection port using a positive displacement pipette. The hybridization protocol was run overnight for 16 h at 48 °C (Appendix F). At termination of the hybridization, microarray slides were removed from the chambers and replaced with blank slides to begin the final drying protocol. Microarray chambers were cleaned with Milli-Q water, placed in a Tupperware container and allowed to air dry in the dark.

2.2.14 Scanning

Slides were covered with an ozone protection barrier and placed in a cassette that was positioned into the Agilent high resolution scanner (Agilent, Mississauga, ON). The scan region was set to 61 mm X 21.6 mm to include the dual array and scan resolution was set at 5 µm. The scanner used two lasers, SHG-YAG laser emitting at 533 nm and a helium neon laser emitting at 633 nm that pass over the slide to give a signal from the green and the red channels. Fluorescence was detected by a photo multiplier tube (PMT) with values set at 100% for both channels. The resulting electrons were captured and processed to produce tagged image file format (TIFF) images that were captured with Agilent scan control software.

2.2.15 Expression data acquisition and flagging

Agilent's Feature Extraction 11.0 program (Agilent, Mississauga, ON) was used to extract expression information from the TIFF image. Feature Extraction grid files were created based on information in the corresponding GenePix array file (GAL) (top array or bottom array). The resulting extraction information was presented in a text file containing expression data. Identification of poor quality spots was based on Cy3 and Cy5 feature saturation, feature pixel variance, background pixel variance and statistical significance of the feature signal compared to the background. Feature Extraction data underwent local background subtraction, LOWESS normalization and expression ratios were $\log_{10}$ transformed.

2.2.16 Microarray data analysis

Feature Extraction processed data was analyzed using Agilent GeneSpring 12 (Agilent, Mississauga, ON) microarray analysis software. Individual array expression data was normalized with LOWESS while between arrays, normalization was performed using a median baseline transformation method, and expression ratios were $\log_2$ transformed. Genes were filtered based on good spot quality, a fold change >1.5 in gene expression and with significance of p-value 0.05. Genes identified as significantly differentiated were analyzed visually using hierarchical and k-means clustering methods that clustered genes based on expression profiles. BLAST2GO (Conesa et al., 2005) was used to retrieve functional annotations for genes identified as significant. Genes that were identified previously during array development and had no associated Gene

Ontology (GO) terms were also considered when analyzing significantly differentiated genes.

2.2.17 Identification of reference gene candidates and genes of interest

Reference gene candidates were identified from the microarray data. Genes having p-value > 0.50, low % CV, fold change < 1.5 and standard deviation < 0.30 were considered stable throughout all endosulfan concentrations. Nine genes of biological interest were selected for RT-qPCR microarray validation. Where possible, forward and reverse primers were designed such that the amplified region would contain the oligonucleotide sequence used for the microarray probe. Primers were designed for the reference and GOI using Integrated DNA Technologies (IDT) PrimerQuest (http://www.idt.com/scitools/applications/primerquest/).

2.2.18 Primer design

The probe sequence of the selected gene was run through BLASTn (http://blast.ncbi.nlm.nih.gov/) to identify all possible ESTs. ESTs that had high levels of accordance with the identity of the probe sequence and a low e-value were selected and aligned using Molecular Evolutionary Genetics Analysis (MEGA) 5 (Tamura et al., 2011). The most representative EST was selected as the template for the PCR primer design. Linearity and the potential for secondary structures were verified using DINAMelt (http://mfold.rna.albany.edu/?q=DINAmelt). IDT PrimerQuest (http://www.idt.com/scitools/applications/primerquest/) was used to design primers, to

ensure that the probe sequence was included in the amplicon region if possible and having a primer annealing temperature of 60 ± 2 °C. To verify specificity, BLASTn (http://blast.ncbi.nlm.nih.gov/) searches were performed on both forward and reverse primers to ensure primers were not similar to any other sequences. Primers were synthesized by IDT (IDT, Coralville, IA) with an oligonucleotide concentration of 0.25 µM, Na^+ concentration of 50 mM and shipped dry. Lyophilized primers were re-suspended with nuclease free water to a working concentration of 10 µM.

2.2.19 cDNA synthesis

RNA extracts used for the microarray hybridizations were converted to cDNA using Invitrogen SuperScript® III First Strand Synthesis SuperMix (Invitrogen, Burlington, ON). One micro-gram of total RNA was added to 1 µl of oligo-DT primer, 1 µl of annealing buffer and topped up to 8 µl with nuclease free water. The solution was mixed thoroughly by manual flicking, vortexed and incubated at 65 ºC for 5 min before being placed on ice for 1 min. Ten micro-litres of 2x First Strand Reaction Mix and 2 µl of SuperScript III®/RNase OUT™ enzyme mix were added to the samples, mixed and spun briefly before incubation at 50 ºC for 50 min. The reaction was terminated by heating at 85 ºC for 5 min, followed by incubation on ice. Forty microlitres of nuclease free water was added to each sample and 4 µl aliquots were dispensed and stored at -80 ºC.

2.2.20 RT-qPCR optimization

To determine the optimal annealing temperature of the primers, a temperature gradient was run for each primer set using the Bio-Rad Chromo4 thermocycler (Bio-Rad, Hercules, CA). The PCR cycling protocol was 50 ºC for 2 min, 95 ºC for 2 min followed by 40 cycles of denaturation at 95 ºC for 2 s, an annealing temperature gradient from 55-70 ºC for 20 s and extension at 72 ºC for 20 s. A melt curve was run at the end of the protocol from 65-90 ºC at 1 ºC intervals and held for 3 s per temperature interval. A 20 µl reaction consisting of 10 µl SYBR® GreenER™ (Invitrogen, Burlington, ON) qPCR Supermix, 0.4 µl of forward and reverse primers at 10 µM, 7.2 µl nuclease free water and 2 µl of cDNA template was added to each well. Reactions were carried out in 0.2 ml white tube strips with clear lids or white 96 well plates with microseals. Fluorescence was detected by using the Opticon Monitor 3 (Bio-Rad, Hercules, CA) software package.

2.2.21 RT-pPCR primer product verification by gel electrophoresis

PCR primer products were electrophoresed in a 2% agarose gel. The gel was made by weighing out the desired amount of agarose and the appropriate volume of 1×Tris/Borate/EDTA (TBE) buffer. The solution was microwaved in short intervals and mixed well during a 2 min period. The solution was cooled after which 15 µl of SYBR® Safe (Invitrogen, Burlington, ON) gel stain was added to the warm agarose solution. The agarose was poured into the gel loading tray containing a well comb and was solidified after 15-20 min at ambient room temperature. The well comb was removed and the tray was filled with 1× TBE. Seven microlitres of gel loading dye was added to

20 µl of PCR product and 13 µl of the solution were carefully loaded into a well. Thirteen microlitres of a standard 1 Kb gel electrophoresis ladder (BioShop, Burlington, ON) were also added to at least two wells on the gel. The power supply (Bio-Rad) was set to the appropriate voltage and run for 20-45 min, depending on the size of the gel. The gel was removed from the tray and an image was acquired using a Bio-Rad Versa Doc (Bio-Rad, Hercules, CA).

2.2.22 RT-qPCR efficiencies

After an optimal annealing temperature was determined, primer efficiencies were performed in triplicate. A three step cycling protocol was used with primer specific annealing temperatures (Appendix G) and each protocol had a melt curve analysis from 65-90 ºC with fluorescent readings at each 1 ºC to ensure the reaction produced only one amplicon. PCR reactions were carried out with SYBR® GreenER™ qPCR Supermix (Invitrogen, Mississauga, ON) as detailed above. A 5× serial dilution of cDNA was used to create a standard curve and the efficiencies were calculated where $E = 10^{(-1/slope)}$ (Hellemans et al., 2007). The efficiencies for all primers were between 89.0 and 106.33% with r^2 between 0.985 and 0.999 that were indicative of high quality performance of the primers.

2.2.23 RT-qPCR analysis of endosulfan exposed samples

Samples used for RT-qPCR validation were run over two plates. The first plate contained 32 samples that were run in triplicate. The second plate contained the

remaining 4 samples, no reverse transcription (RT) control, no RNA control and 3 inter-run calibrator (IRC) samples to remove any inter-run variation, all plated in triplicate. The SYBR® GreenER™ (Invitrogen, Burlington, ON) qPCR reaction mix was made as described previously (2.2.20) and loaded into the QIAgility (Qiagen, Germantown, MD) PCR setup machine along with 40 µl of cDNA per sample. The QIAgility was programmed to add 13 µl of master mix and 2 µl of cDNA to each well of the 96 well plate. Plates were covered with a PCR plate microseal, placed in the Bio-Rad Chromo4 thermocycler (Bio-Rad, Hercules, CA) and the appropriate 3-step protocol was selected.

RT-qPCR results were analyzed with Biogazelle qbase^{PLUS} (Biogazelle, Ghent, Belgium) software and previously defined target specific amplification efficiencies were used for quantification. Replicate variability, removed samples where the difference in Ct value was > 0.5 and the negative control threshold, identified as the minimum difference between the control sample and the sample with the highest sample Ct value, was set to 5.

The stability of 11 reference genes was measured in all 36 samples. Quality control settings for reference target stability were geNorm M <1.25 and V <0.15. Reference target genes hypothetical protein (Hyploc), conserved hypothetical protein (CHP), Reference 301 (R301), Reference 411 (R411) and Brefeldin A (Bref) were identified as the most stable; the normalization factor (NF) was identified as the geometric mean of the reference target genes. The subsequent normalization resulted in normalized relative quantities (NRQ). Samples were run over two PCR plates; therefore the calibration factor (CF) was calculated for each GOI based on the IRCs. The CF was applied to the NRQ value resulting in calibrated normalized relative quantities (CNRQ) (Vandesompele et al., 2002).

2.2.24 Statistical analysis

At termination of the exposure experiment, survival and mortality data was analyzed using TOXSTAT probit (adapted from Stephan, 1977) analysis software to calculate an LC_{50} with a 95% confidence interval. Microarray gene expression values were analyzed using a 1-way ANOVA and 100 permutations to detect significance at p-value <0.05. A Tukey post hoc test was also used to identify significantly differentiated genes between endosulfan concentrations. The mean CNRQ values from the RT-qPCR analysis of GOI for each concentration were compared to identify significant differences in relative abundance using a 1-way ANOVA with multiple test corrections and significance at p-value < 0.05. The CNRQ values were $\log_2$ transformed and compared to microarray $\log_2$ transformed expression ratios.

2.3 Results

2.3.1 Water chemistry analysis

Water chemical analysis was performed at Environment Canada Atlantic Laboratory for Environmental Testing (ALET) in Moncton, NB. Fresh and aged endosulfan water samples were analyzed at the beginning, middle and near the end of the chronic exposure by gas chromatography electron capture detector (GC-ECD). Measured experimental concentrations were significantly lower than the nominal concentration with average recoveries between 12.5-55.5%. The concentration of the endosulfan metabolites α- endosulfan, β- endosulfan and endosulfan sulfate were

measured and summed to determine the total amount of endosulfan extracted (Table 2.4). Although the nominal concentrations of 0 (0), 0.01 (0.005), 0.03 (0.008), 0.1 (0.021), 0.3 (0.5) and 1.0 (0.26) μg/L had much lower experimental values, the gradient of a low to high concentration was maintained and control samples had metabolite concentrations below the minimum detection limit (MDL) of 0.00025 μg/L in 4 L. Aged samples (5-7 days old) had considerably lower concentrations of all metabolites compared to fresh samples indicating the degradation of endosulfan over time. The total endosulfan degradation between fresh and aged solutions was 39-77%. The presence of endosulfan sulfate in fresh samples was below the MDL; however levels were 4-13% higher in aged samples.

The observed negative effects were as expected and therefore the lobster larvae may be sensitive to endosulfan concentrations lower than previously thought. The reasons for the significantly lower measured concentrations are unknown despite precautions being taken to ensure the integrity of the water chemistry samples. The concentrations referenced throughout the remainder of the thesis are the average measured concentrations and not the nominal concentrations.

Table 2.4: The average total amount of endosulfan recovered from water chemistry analysis by GC-ECD. Endosulfan metabolites α-endosulfan, β-endosulfan and endosulfan sulfate were summed to determine the total endosulfan concentration in fresh and aged solutions with a minimum detection limit of 0.00025 µg/L in a 4 L sample for each metabolite.

Nominal Concentration (µg/L)	Fresh Solution Measured Concentration (µg/L)	Aged Solution Measured Concentration (µg/L)
0	<0.0008	<0.0008
0.01	0.005	0.0015
0.03	0.008	0.0019
0.1	0.021	0.0040
0.3	0.05	0.0116
1.0	0.26	0.1307

2.3.2 The effect of endosulfan exposure on larval survival and development

Traditional toxicology endpoints and novel microarray analysis of gene expression were used to monitor the sublethal effects at an organismal and molecular level. After the 14 day chronic exposure, there were high levels of mortality at 0.021, 0.05 and 0.26 μg/L and decreased levels of mortality in the lower concentrations and the control (Figure 2.1). The LC_{50} of the chronic exposure was calculated to be 0.12 μg/L (0.066-0.3).

Animals that survived the exposure concentrations of 0.021, 0.05 and 0.26 μg/L showed signs of developmental delays and lethargy. At higher endosulfan concentrations, fewer animals progressed through the metamorphic moult resulting in higher number of stage III than stage IV postlarvae. Interestingly, there was also a visible trend in the number of days required to reach stage IV. As the concentration of endosulfan increased, there was an increase in the number of days required to reach metamorphosis (Table 2.5). The majority of animals remaining in the lower concentrations were stage IV postlarvae that had successfully undergone metamorphosis.

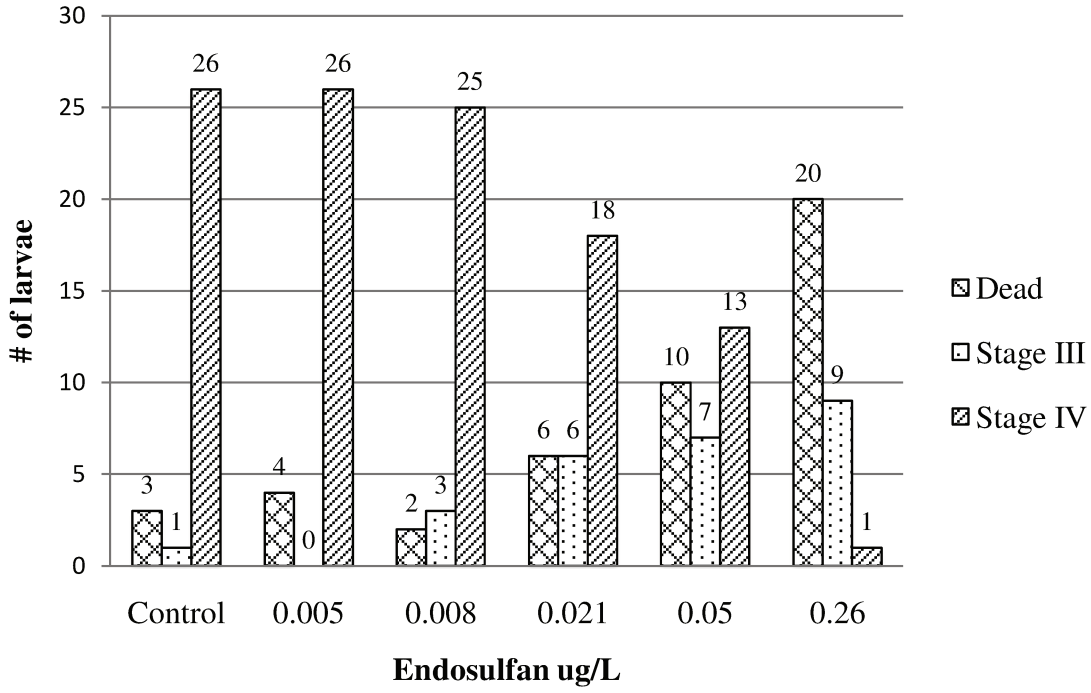

Figure 2.1: The effects of endosulfan on survival and development during H. americanus larval metamorphosis. Cumulative mortality and the number of stage III and stage IV larvae remaining in each endosulfan concentration (0, 0.005, 0.008, 0.021, 0.05 and 0.26 µg/L) at termination of the 14 day exposure experiment were shown above the respective bar.

Table 2.5: The average number of days required for H. americanus larvae to reach metamorphosis. The average number of days required to reach metamorphosis was calculated from the surviving larvae of each endosulfan concentration (0 μg/L, n=27; 0.005 μg/L, n=26; 0.008 μg/L, n=28; 0.021 μg/L, n=24; 0.05 μg/L, n=20 and 0.26 μg/L, n=10). Significance was determined by 1-way ANOVA at p-value < 0.05.

Concentration (μg/L)	Average # of days ±SD
0	8.63 ± 1.62
0.005	8.73 ± 1.40
0.008	9.61 ± 1.93
0.021	10.15 ± 2.18 *
0.05	12.00 ± 2.08*
0.26	+ 14 *

2.3.3 Microarray analysis

A total of 707 genes were determined to be statistically differentially expressed in endosulfan exposed H. americanus larvae. A Tukey's post hoc test was performed to identify the number of differentially expressed genes among 15 pairwise comparisons, with the primary interest being genes showing significant change from control gene expression levels. Briefly, the 0.26 µg/L exposure group had 352 genes significantly different from the controls followed by 0.05 µg/L exposure group with 87, which were the concentrations where there was increased mortality and developmental delays during the exposure experiment. The 0.005, 0.008, and 0.021 µg/L endosulfan exposure groups had far fewer significantly differentiated genes of 25, 11 and 14 respectively. A total of 489 genes were identified as significantly differentiated between the control and at least one exposure group, while the remaining 218 genes showed differentiation between endosulfan exposure groups.

The 707 genes were clustered using a hierarchical clustering method that further confirmed an increase in significantly expressed genes at the higher concentrations (Figure 2.2). K-means cluster analysis was performed to categorize interesting and unique expression profiles wherein 4 clusters were identified to best represent the data (Figure 2.3). The oligonucleotide sequences of statistically relevant genes were uploaded into BLAST2GO (Conesa et al., 2005) to search for functional annotation based on previously described proteins in Gene Ontology. The BLASTx query was used to search translated nucleotide sequences in a protein sequence database. Among the 707 genes of interest, only 185 (26%) were functionally annotated, with the majority of genes involved in metabolic and cellular processes and biological regulation.

2.3.4 K-Means analysis of significantly differentiated genes

Significantly differentiated genes were separated into 4 clusters using a k-means clustering algorithm. Although only a small portion of significant genes were annotated, the functional groups were diverse across clusters. Genes were categorized into general biological processes such as development, metabolism, gene regulation, immune and oxidative stress response. Subsequent to the low number of functionally annotated genes, there were not always descriptive representatives from each biological process within each cluster.

The expression profile of cluster 1 demonstrated an initial low level of expression in the controls through to 0.008 µg/L exposure group. At the 0.021 µg/L, expression levels rose drastically until the 0.05 µg/L when the expression droped sharply at the 0.26 µg/L exposure group to levels similar to the controls. There were 95 genes assigned to cluster 1 of which only 40% were functionally annotated (Appendix H1). Many of the genes in this cluster were involved in gene regulation (n = 10) and metabolism (n = 7) with very few genes involved in immune and oxidative stress response or development (n = 1 and n = 2, respectively). Genes involved with gene regulation were often associated with transcription, translation or protein binding, whereas genes related to metabolism varied greatly in their function.

The 253 genes identified in cluster 2 (Appendix H2) had low expression levels in the control and 0.005 µg/L exposure group followed by slight up regulation at 0.008 µg/L before returning to lower expression levels at 0.021 µg/L exposure group. Relative expression levels gradually increased in the 0.05 µg/L group before increasing rapidly in the 0.26 µg/L exposure group. As one of the larger clusters, there was still only 38%

functional annotation. The majority of genes identified in this cluster were involved in gene regulation (n = 32), specifically ribosomal associated proteins (n=25). Among the genes involved with metabolism (n = 9), many such as electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial trifunctional protein beta subunit and NADH-ubiquinone oxidoreductase were all associated with the mitochondria and the electron transport chain (ETC). Genes related to immune and oxidative stress response (n = 6) were DNA repair proteins, ferritin, thioredoxin and a detoxifying enzyme GST. There were very few genes involved in development identified in this cluster (n = 4).

Cluster 3 had an interesting expression profile demonstrating low expression levels in the control group followed by steady increase in expression to the 0.05 μg/L exposure group and then decreasing to very low expression levels at the highest concentration. Among the 104 genes identified in cluster 3, 43% were annotated; the highest annotation level of all the clusters (Appendix H3). There were a greater number of genes involved with development and metabolism (n = 9 and n = 12 respectively) compared to gene regulation (n = 5) and immune and oxidative stress response (n = 3). Interestingly, several of the developmental genes identified were related to the integrity of the exoskeleton or genes expressed in primordial germ cells (PGC) such as the zinc knuckle protein and DEAD box ATP-dependent RNA helicase.

Finally, cluster 4 had an expression profile showing low levels of relative expression in the control through to the 0.021 μg/L exposure group followed by rapid increase in expression at the 0.05 μg/L and 0.26 μg/L exposure groups. The numbers of genes involved in gene regulation and metabolism were similar (n = 23 and n = 20) as were genes involved in development and immune and oxidative stress response (n = 10 and n = 11). This was the largest cluster with 255 genes and 41% annotation (Appendix

H4). Interestingly, 5 of 9 GOI were placed in cluster 4 such as fushi tarazu F1 (FTZ-F1) transcription factor, cuticle protein, glutathione-S-transerfase (GST), farnesoic acid o-methyltransferase (FAO-MeT) and Histone H1. Half of the development and immune and oxidative stress response genes identified in the data set were also found in this cluster.

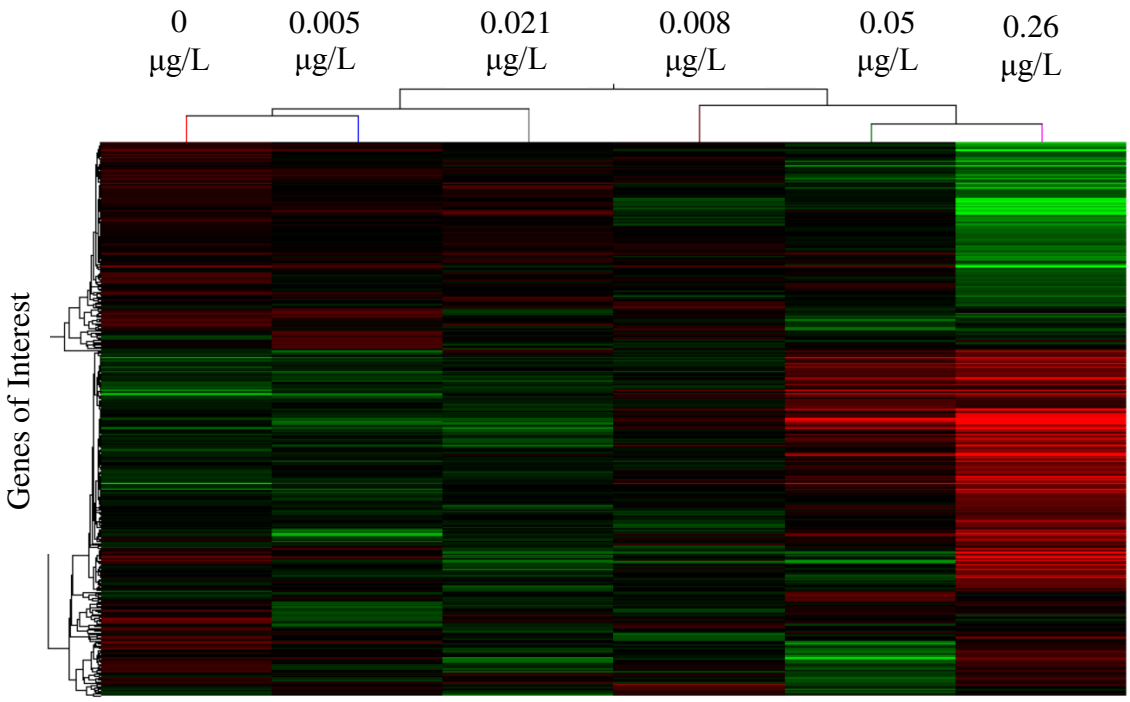

Figure 2.2: Hierarchical cluster of 707 significantly differentiated genes identified by microarray analysis in H. americanus larvae exposed to endosulfan. The average inverse $\log_2$ ratios (Cy5/Cy3) were clustered where green indicated up regulation in endosulfan exposed samples and red indicated down regulation. The endosulfan concentrations were clustered on the x-axis and significant genes were clustered on the y-axis. Significant genes were identified as having a fold change ≥ 1.5 and p-value < 0.05 after 1-way ANOVA and permutation tests.

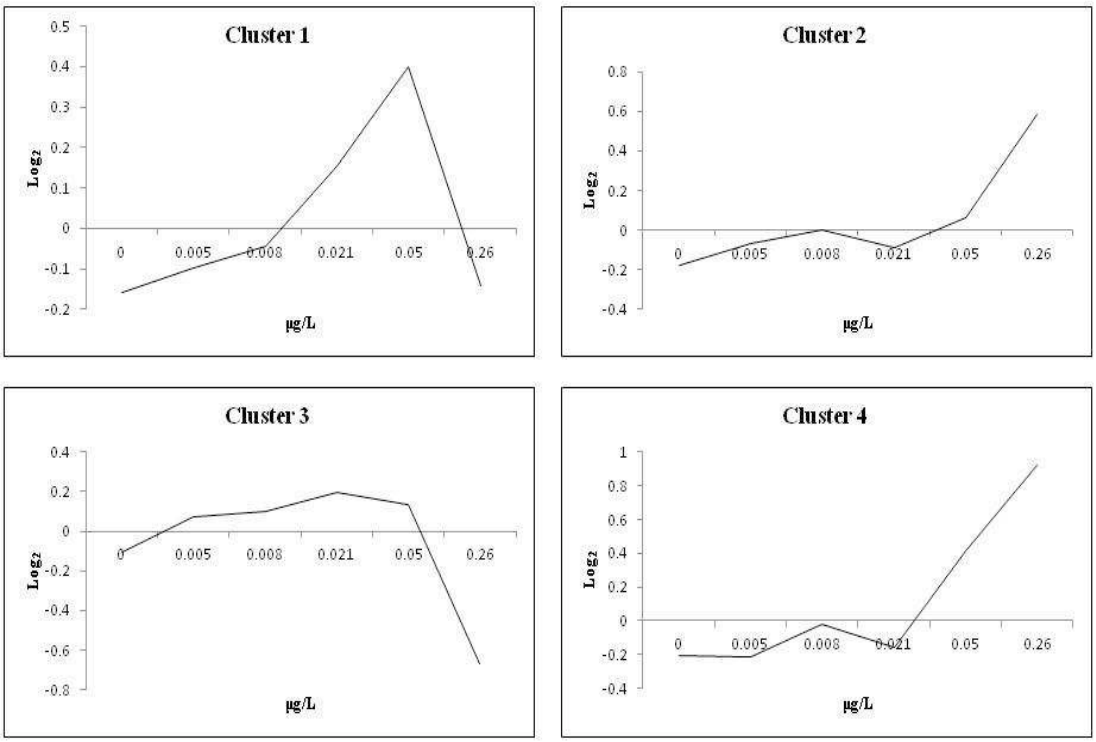

Figure 2.3: The k-means cluster profiles for significantly differentiated genes from H. americanus larvae exposed to endosulfan. Profiles were the average inverse $\log_2$ expression values of genes at each endosulfan exposure concentration (0, 0.005, 0.008, 0.021, 0.05 and 0.26 µg/L). Positive values were indicative of up regulation and negative values were indicative of down regulation. Each panel was labelled with the corresponding cluster number **A**: Cluster 1, **B**: Cluster 2, **C**: Cluster 3 and **D**: Cluster 4.

2.3.5 RT-qPCR microarray validation

Genes of interest (GOI) were selected for validation of the microarray results using RT-qPCR analysis. In total, 9 GOI with candidates from development, metabolism, gene regulation and immune and oxidative stress response processes were chosen. The expression pattern found in the RT-qPCR results were similar to that of the microarray in identifying up and down regulated genes; however the intensity of expression was often significantly different between the two methods. In 7 of the 9 GOI, the RT-qPCR results showed increased sensitivity in detecting the expression level within each of the concentrations compared to the microarray results. Ecdysone and NADH dehydrogenase were the 2 GOI in which the microarray detected greater fold changes compared to RT-qPCR (Figure 2.4).

Among the 9 genes chosen for RT-qPCR microarray validation all except ecdysone, were identified as being significantly differentiated in the analysis of the microarray data between at least one endosulfan concentration and the control group. The RT-qPCR method identified Histone H1, thioredoxin, cuticle protein, FAO-MET and GST as significantly differentiated between the control and 0.26 μg/L exposure group in addition to the cuticle protein, FAO-MET and GST significant at the 0.05 μg/L group. Despite NADH dehydrogenase, FTZ-F1 and ferritin being identified as significant at the 0.26 μg/L exposure group in the microarray analysis, they were not significant in the RT-qPCR validation (Figure 2.4). The expression of ecdysone was the only GOI found not to be significantly differentiated in either the microarray or RT-qPCR method and was selected as a GOI based on functional interest, not on statistics.

Eleven candidate reference genes were measured in each sample during RT-qPCR analysis. The geNorm application in qbase^{PLUS} (Biogazelle, Ghent, Belgium) identified 5 genes: R301, R411, Hyploc, CHP and Bref (Appendix G) to be the most stable and meeting the selection criteria of M value < 1.25 and V value ≤ 1.5 (0.149). In addition, all relative abundance levels obtained through RT-qPCR were expressed as CNRQ values after calibration factor adjustments.

2.3.6 Microarray and RT-qPCR method correlation

Ideally, microarray and RT-qPCR should correlate well in the identification of gene expression profiles. The inverse $\log_2$ transformed microarray ratios and $\log_2$ transformed RT-qPCR relative abundance values for each sample were plotted on a per gene basis to determine the correlation between the two methods (Figure 2.5). Histone H1, GST and the cuticle protein had the highest level of correlation with correlation coefficients of 0.61, 0.54 and 0.56 respectively. The remaining plots indicated very poor correlation between microarray and RT-qPCR results with correlation coefficients all below 0.2.

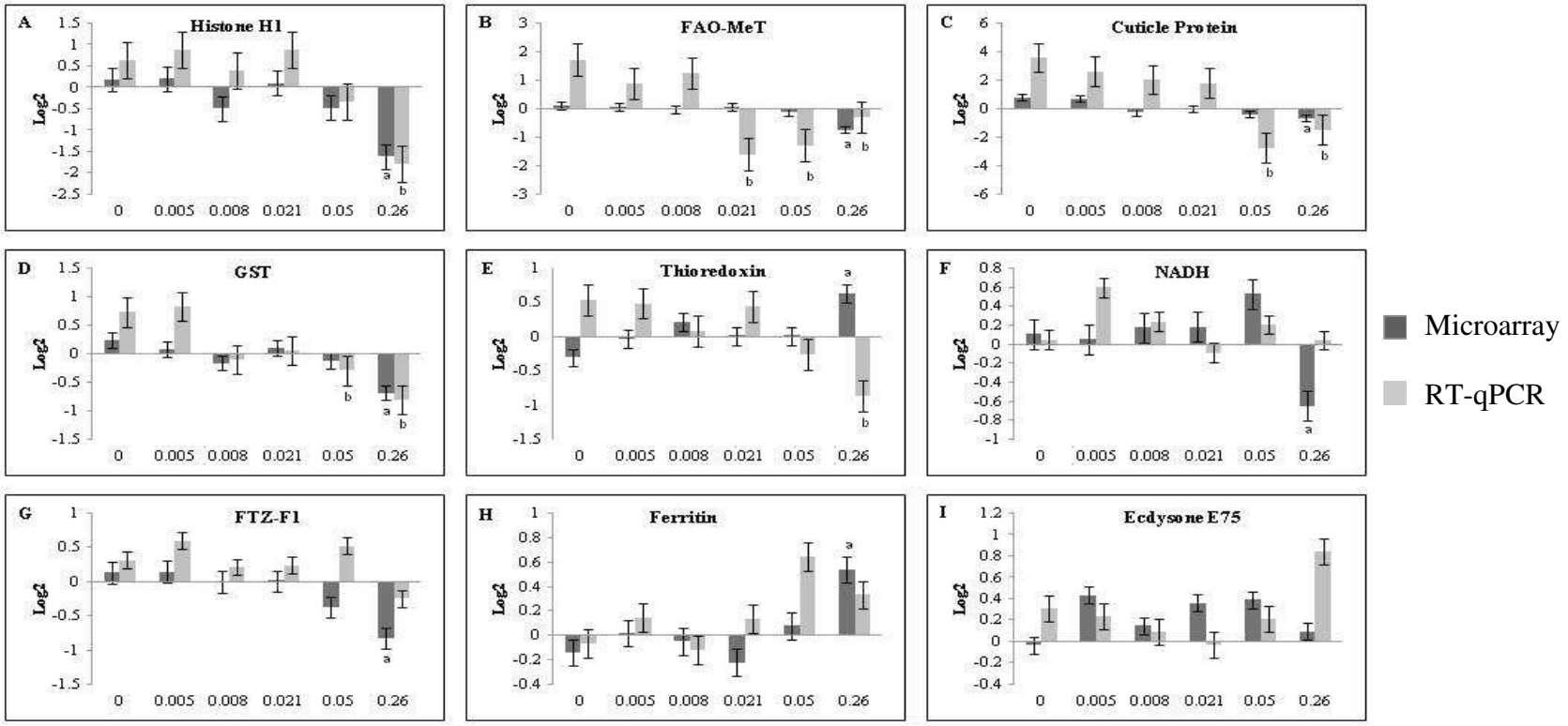

Figure 2.4: The average expression levels of GOI from both microarray and RT-qPCR analysis. Inverse $\log_2$ microarray ratios (Cy5/Cy3) and RT-qPCR $\log_2$ relative abundance values were plotted for each gene of interest. A 1-way ANOVA statistical analysis test and permutations were applied to each data set separately to identify significantly differentiated genes. Significance was identified with p-value <0.05 and was only indicated on the graph if the significance was between the control and an endosulfan concentration(s). Statistically significant genes identified between the control and an exposure group from the microarray and RT-qPCR results are indicated by a and b, respectively. Positive values indicated up regulation whereas negative values indicated down regulation. Each panel was labelled with the corresponding gene of interest: **A**: Histone H1, **B**: FAO-MeT, **C**: Cuticle Protein, **D**:GST, **E**: Thioredoxin, **F**: NADH, **G**: FTZ-F1, **H**: Ferritin and **I**: Ecdysone.

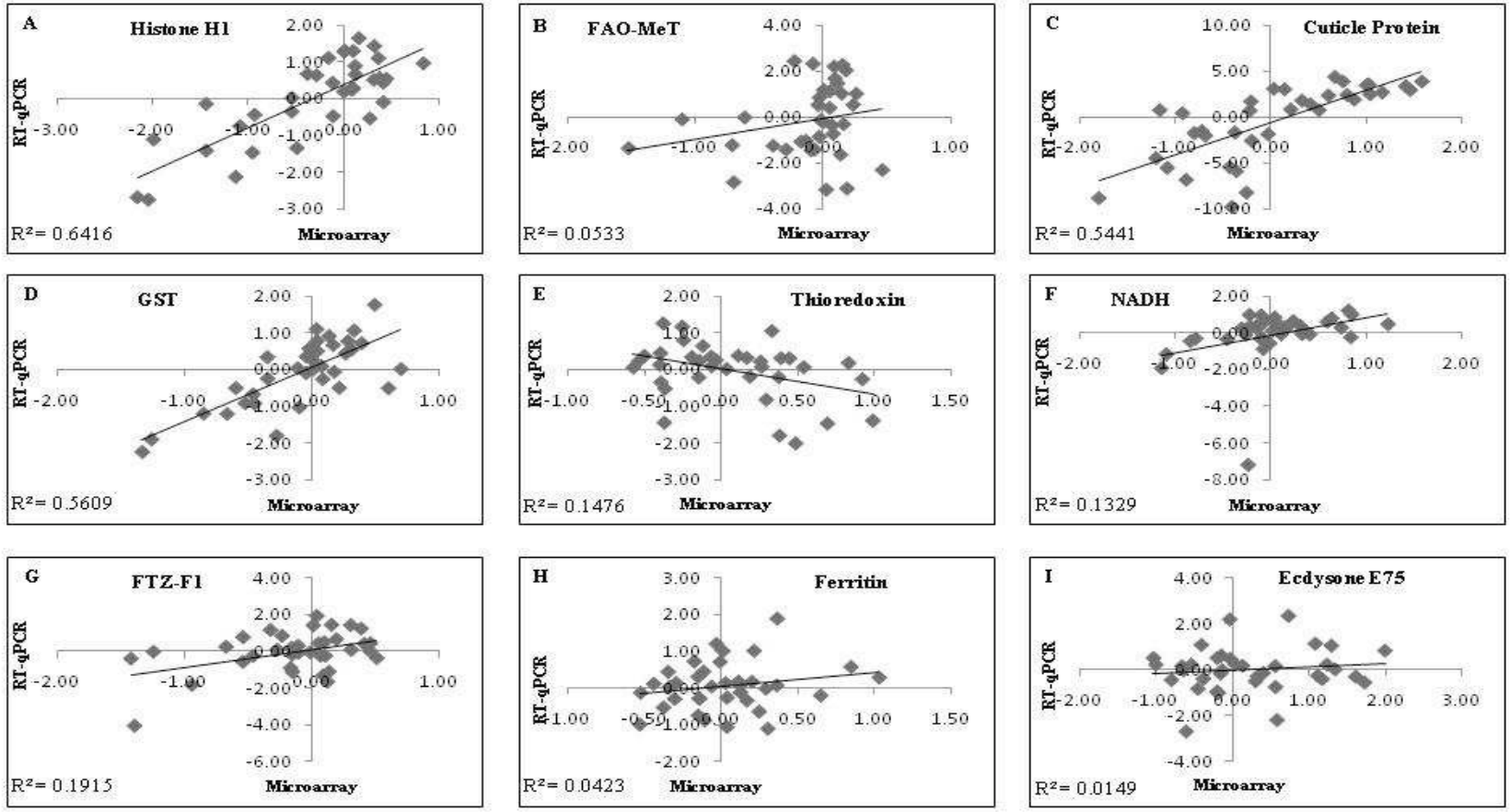

Figure 2.5: The correlation between microarray gene expression and RT-qPCR relative abundance. Inverse $\log_2$ microarray ratios (Cy5/Cy3) and RT-qPCR $\log_2$ relative abundance values were plotted on a per gene basis, a line of best fit was applied and a Pearson correlation coefficient was calculated (r^2). Positive values indicated up regulation and negative values indicated down regulation. Each panel was labelled with the corresponsing gene of interest: **A**: Histone H1, **B**: FAO-MeT, **C**: Cuticle Protein, **D**: GST, **E**: Thioredoxin, **F**: NADH, **G**: FTZ-F1, **H**: Ferritin and **I**: Ecdysone.

2.4 Discussion

Endosulfan is a commonly used agricultural pesticide that acts as a GABA chloride channel inhibitor preventing hyperpolarization. A disruption in repolarization can result in hyperexcitation, convulsion and death of an organism in severe cases (Bloomquist, 2003). The focus of this study was the effects of chronic endosulfan exposure on survival, development and gene expression in H. americanus larvae during metamorphosis.

The progression of larva through metamorphosis to become a postlarva is required for normal development in H. americanus and other crustaceans. During metamorphosis, larval characteristics begin to transform into adult characteristics with significant changes in morphology, physiology, metabolism and behaviour (Anger, 2006; Ennis, 1995) and consequently, suboptimal environmental conditions can have serious effects on this process. The American lobster is an important economic species in Atlantic Canada and exposure to agricultural pesticides could adversely impact the health and recruitment of the lobster population. Although there have been previous studies on the effects of pesticides, petroleum and metals on lobster larva development (Burridge et al., 1999; Derby and Capuzzo, 1984; Marino-Balsa et al., 2000), the current study was able to identify the effects of endosulfan using traditional toxicology endpoints and changes at the gene expression level.

2.4.1 Water chemistry analysis

Water chemical analysis was successful in identifying endosulfan metabolites; however the measured concentrations were significantly lower than nominal concentrations in fresh samples (Table 2.4). The reasons for the significantly lower measured concentrations are unknown despite precautions to ensure the integrity of the water chemistry samples. Some possible explanations for the lower concentrations are inaccurate test solution concentration, loss during water transfer and loss during water chemical analysis. The concentration of endosulfan sulfate was below the MDL in fresh samples whereas elevated concentrations were found in aged samples, confirming that endosulfan sulfate is a major metabolite of endosulfan degradation (Awasthi et al., 2000; Guerin, 2001; Hoang et al., 2011; Raupach et al., 2001; Weber et al., 2010). Despite the increase in endosulfan sulfate in aged samples, α- endosulfan was still present at the highest concentration of all the metabolites in aged samples, which is consistent with the order of endosulfan metabolites found in the environment of $\alpha > \beta >$ sulfate (Weber et al., 2010).

2.4.2 Endosulfan 14 day chronic exposure

Increased mortality was observed at higher endosulfan concentrations, which was likely attributable to the neural inhibition effects of endosulfan and surviving animals showed signs of developmental abnormalities. The exposure of various species to endosulfan has caused developmental delays in the common toad (Bufo bufo) (Brunelli et al., 2009), fruit fly (Drosophila melanogaster) (Sharma et al., 2011), streamside salamander (Ambystoma barbouri) (Rohr et al., 2003) and the water flea

(Daphnia magna) (Zou and Fingerman, 1997), changes in reproduction and development in Japanese medaka (Oryzias latipes) (Gormley and Teather, 2003) and zebra fish (Danio rerio) (Han et al., 2011), metabolic changes in speckled shrimp (Metapenaeus monoceros) (Suryavanshi et al., 2009) and fresh water catfish (Clarias batrachus) (Tripathi and Verma, 2004), and changes in gene expression in both the fiddler crab (U. pugilator) (Meng and Zou, 2009) and D. melanogaster (Sharma et al., 2011).

The 14 day chronic endosulfan exposure resulted in increased mortality in the 0.05 and 0.26 μg/L exposure groups and a significant delay in the number of days required to reach metamorphosis in the 0.021, 0.05 and 0.26 μg/L groups (Table 3.1). The Canadian Water Quality Guideline for marine water at the time of the study was 0.02 μg/L (CCREM, 1987), however it has since been lowered to 0.002 μg/L (CCME, 2010). Waterborne contaminant levels for marine environments in Atlantic Canada are scarce, however monitoring performed by Environment Canada has detected endosulfan levels of 0.05 μg/L in freshwater bodies in New Brunswick (Christine Garron, Environment Canada, pers. communication), indicating that larvae in the wild could be exposed to elevated endosulfan concentrations. Endosulfan is considered to be a persistent organic pollutant (POP) with a half-life varying between a few hours and a year depending on the environment (water, soil or air) and environmental conditions (Weber et al., 2010). The results of this study confirm that sublethal exposure of lobster larvae to endosulfan impacts both development and survival (Figure 2.1 & Table 2.1). Larval stages in many species have been identified as vulnerable to environmental stressors (Brunelli et al., 2009; Burridge et al., 1999; Derby and Capuzzo, 1984; Ennis,

1995; Marino-Balsa et al., 2000). The results of this study indicate that endosulfan is detrimental to developing lobster larvae.

2.4.3 Microarray

The microarray analysis of surviving endosulfan exposed larvae resulted in the identification of 707 genes that were considered to be statistically differentiated. Despite a relatively low annotation level of the lobster microarray, ~ 40%, genes of biological interest involved in different processes were pursued for further consideration to better understand what effects endosulfan may have on development, metabolism, immune and oxidative stress response and gene regulation.

2.4.4 Developmental effects of endosulfan

Metamorphosis is an important developmental event where lobster larvae undergo significant morphological changes to prepare for the benthic environment. Wingless (wg) is part of the Wnt gene family that is involved in many developmental processes such as segment polarity, neural development and axis formation in Drosophila (DasGupta et al., 2005). Pontin, a Wnt antagonist, can cause disruptions in Wnt signaling, specifically the β-catenin pathway involved in cell differentiation and organogenesis (Corada et al., 2010; DasGupta et al., 2005; Nusse and Varmust, 1992). Wingless-type and Pontin were clustered together (cluster 2) suggesting that endosulfan may impact these important developmental signaling pathways. Although surviving postlarvae did not exhibit any morphological deformities, they have been seen in D.

melanogaster (Sharma et al., 2011), B. bufo tadpoles (Brunelli et al., 2009) and D. rerio larvae hatched from endosulfan exposed adults (Velasco-Santamaría et al., 2011). Further histology samples would be required to determine if there were any deformities at the tissue or cellular level in the lobster larvae.

Endosulfan had significant effects on moulting and metamorphosis in H. americanus larvae. Moulting is required for normal growth and development by which the hardened exoskeleton is shed to allow for increased size and volume. At the gene expression level, several genes involved in cuticle formation such as chitinases, dumpy and cuticular proteins were all down regulated at 0.26 µg/L endosulfan concentration.

Chitinases are categorized into two glycoside hydrolase (GH) families GH family 18 and 19 (Ubhayasekera, 2011). GH family 18 chitinases occur in a diverse group of organisms from bacteria to mammals and have a wide array of functions such as immune response, embryonic development and chitin degradation, depending on the species in which they are found (Ubhayasekera, 2011; Huang et al., 2012). The number of chitinases in the GH family 18 is also species dependent, however 35 have been identified in the nematode (Caenorhabditis elegans), 17 in the fruit fly (D. melanogaster) and 3 in speckled shrimp (M. japonicas) (Huang et al., 2012; Proespraiwong et al., 2010). Chitinases are chitinolytic enzymes that are important for moulting; they bind to chitin and break it down into N-acetylglucosamine monomers (Huang et al., 2012; Meng and Zou, 2009; Proespraiwong et al., 2010; Zou and Bonvillain, 2004). Although the mechanism by which endosulfan delays moulting is unknown, chitinolytic enzymes are the end product of the moulting hormone cascade and are useful indicators for determining if environmental contaminants elicit effects on moulting (Meng and Zou, 2009).

The cuticle is composed of 4 different layers, the epicuticle, exocuticle, endocuticle and a membranous layer, all of which contain proteins of various sizes (O'Brien et al., 1991). Cuticular proteins are the structural building blocks for the cuticle interacting with chitin and calcium to produce the hardened exoskeleton (Mary and Krishnan, 1974). Analysing the properties of cuticular proteins such as size, polarity solubility and isoelectric points has been useful in determining difference in cuticular proteins between the 4 layers and what effect this has on cuticle formation and function (Mary and Krishnan, 1974; O'Brien et al., 1991). The down regulation of cuticular proteins after endosulfan exposure is therefore suspected to have serious effects on development and integrity of cuticle in the lobster larvae.

Dumpy, an extracellular protein, is responsible for changes in growth, morphogenesis and cuticle composition in Drosophila (Wilkin et al., 2000). Normal cuticle development is important to maintain shape, function and overall growth and development (Wilkin et al., 2000). The down regulation of chitinases, dumpy and cuticular proteins provides compelling evidence that endosulfan has detrimental effects on the moult hormone signaling pathway and cuticle formation. The changes in expression of these genes correlate well with the results observed throughout the exposure experiment where there was a decrease in the number of animals moulting to stage IV in higher endosulfan concentrations.

At termination of the endosulfan exposure, there was a significant increase in the number of days required to reach metamorphosis and a subsequent delay in moulting. In crustaceans, moulting is a hormonally regulated process involving the moult inducing hormone ecdysone, moult inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH). Methyl farnesoate (MF) is considered equivalent to the insect juvenile

hormone (JH), which is also involved in moulting (Abdu et al., 1998; Borst et al., 1987; Felterman and Zou, 2011; Laufer and Biggers, 2001). Significant changes in gene expression were seen in moult related genes such as FTZ-F1 and FAO-MeT, after endosulfan exposure. Ecdysone elicits a response when the ecdysone receptor (EcR) heterodimerizes with ultraspiracle (USP) and this receptor complex regulates a few early response target genes (Pierceall et al., 1999). These primary target genes in turn regulate the ecdysone signal and affect the expression of hundreds of secondary genes (Sullivan and Thummel, 2003).

Research on nuclear receptors expression during development in Drosophila has determined that many receptors are tightly linked and are involved in metamorphosis including EIP-75 and FTZ-F1 (Dubrovsky et al., 2011; Pierceall et al., 1999; Sullivan and Thummel, 2003). FTZ-F1 is considered to be regulated downstream of direct ecdysone signaling as an increase in FTZ-F1 has been seen after increased ecdysone titres and E75 up regulation (Bialecki et al., 2002; Sullivan and Thummel, 2003). In Drosophila, the temporal expression of nuclear receptors was monitored in embryos through to metamorphosis, and was determined that there was reoccurring expression patterns among E75, FTZ-F1 and a Drosophila hormone receptor 3 (DHR3) (Sullivan and Thummel, 2003). The reciprocal gene expression pattern of FTZ-F1 and E75 in this study supports the findings of similar patterns in the tobacco horn worm (Manduca sexta) (Riddiford et al., 2003). FTZ-F1 is involved in the development of the cuticle, and its temporal expression is critical for normal cuticle development (Sullivan and Thummel, 2003; Yamada et al., 2000). The down regulation of FTZ-F1 at the 0.05 and 0.26 μg/L exposure groups is further support that endosulfan has negative effects on development of new cuticle and ecdysis.

JH is involved with development and the initiation of reproduction in insects; MF is suspected to have similar functions in crustaceans (Borst et al., 1987; Nagaraju, 2007). MF is produced by the methylation of farnesoic acid (FA) by FAO-MeT and there is significant research into MF potential involvement in moulting and onset of metamorphosis (Abdu et al., 1998; Borst et al., 1987; Nagaraju, 2007). Lower levels of JH in insects identify a period of restructuring in preparation for metamorphosis and increased levels signify induction of moult (Dubrovsky et al., 2011; Nagaraju, 2007). JH increases the expression of E75, however it is thought to be mediated by FTZ-F1 that acts as a competency factor (Dubrovsky et al., 2011). The results from the microarray analysis identified down regulation of FAO-MeT in the 0.05 to 0.26 µg/L exposure groups and up regulation in all other concentrations. Interestingly, MF levels in the green crab (Carcinus maenas) increase in response to physiological stress (Lovett et al., 1997), which supports the up regulation of FAO-MeT observed in the lower endosulfan concentrations. The observed decrease in expression at the higher concentrations could indicate that stage III larvae were nearing metamorphosis after significant delay and were preparing to moult.

During the moult cycle, the mobilization of Ca^{2+} is important for the calcification of the newly synthesized exoskeleton. Major calcium fluctuations throughout the moult cycle are regulated by epithelial cells in the gills, gut, antennal gland and the integument (Ahearn et al., 2004). Inside the epithelial cells, organelles such as mitochondria, lysosomes and sarcoplasmic reticulum/endoplasmic reticulum (SR/ER) are involved in controlling Ca^{2+} concentrations in the cytoplasm (Ahearn et al., 2004). The ryanodine receptor is found on Ca^{2+} channels in the SR/ER and controls the outflow of Ca^{2+} into the cytoplasm (Tumburu, 2010). After endosulfan exposure, the ryanodine receptor was

significantly down regulated in the 0.26 µg/L exposure group and could consequently result in changes in Ca^{2+} regulation. Endocrine disrupting chemicals (ECD) affect many biological and physiological signaling pathways. ECD also affect Ca^{2+} regulation, which for crustaceans is crucial for development of the exoskeleton where ~ 80% of Ca^{2+} is stored (Wheatly et al., 2002; Tumburu, 2010). Oxidative stress causes an imbalance of Ca^{2+}, specifically an accumulation of Ca^{2+} in the cytosol resulting in apoptosis (Korsloot et al., 2004; Wang et al., 2009). Changes in Ca^{2+} occur naturally throughout the moult cycle and more research would be needed to determine if the changes observed resulted from moult stage or endosulfan exposure.

Lobster larvae exposed to endosulfan could also have long term effects on reproduction and health of the next generation. Endosulfan exposure showed effects on DEAD-box ATP dependent RNA helicase and the zinc knuckle domain, two genes related to the vasa gene that is important for the development of primordial germ cells (PGC). PGCs are the germ cell precursors that are necessary for sex determination and reproductive success (Sellars et al., 2007). In zebrafish (D. rerio) offspring from endosulfan adults showed lordosis of their spine. Since offspring were not directly exposed to endosulfan, changes to the PGCs could have caused developmental deformities in the offspring (Velasco-Santamaría et al., 2011). Further studies on the reproductive success of H. americanus exposed to endosulfan would be needed to confirm that changes seen in PGC could have negative ramifications on the next generation.

Deviations from normal development are good indicators of stress; however growth and development can be further impaired by decrease in energy allocation caused by changes in metabolism. Under suboptimal conditions, energy may be

reallocated to cope with the stress and maintain basal metabolic processes instead of being allocated for growth and reproduction (Capuzzo et al., 1984; Soetaert et al., 2007).

2.4.5 Metabolic effects of endosulfan

Dietary requirements for H. americanus vary depending on developmental stage, habitat and ability to store nutrients. Larvae living in the pelagic zone require increased levels of protein and lipid since they do not have the ability for significant nutrient storage (Conklin, 1995; Sasaki, 1984; Sasaki et al., 1986). Assuming favourable environmental conditions, developing larvae have the most rapid growth rate of the lobster life cycle and also have increased requirements for protein and lipids (Conklin, 1995; Sasaki, 1984). The need for carbohydrates is considerably lower throughout all life stages and they do not appear to be digested as easily as protein and lipid (Conklin, 1995). A balanced diet and efficient utilization of energy is essential for larvae to successfully proceed through to metamorphosis (Capuzzo et al., 1984; McKenney et al., 1998; Sasaki et al., 1986). Alterations in metabolism can have effects on survival, increase susceptibility to other stressors and decrease growth and development (Capuzzo et al., 1984; Soetaert et al., 2007). Lobster larvae exposed to endosulfan showed increase in expression of AMP dependent CoA ligase, flavoprotein ubiquinone and mitochondrial trifunctional protein, all of which are involved in lipid metabolism. Lobster larvae were starved for a period of ~20 h before collection; lipids are utilized first during short term starvation (Ritar et al., 2003). Also, these animals were late stage III or postlarvae which develop an increased lipid level capacity around metamorphosis as a potential sign of energy storage modification that is indicative of adult stages

(Sasaki, 1984; Sasaki et al., 1986). Grass shrimp (Palaemonetes pugio) and the freshwater field crab (Barytelphusa guerini) exposed to endosulfan had an overall decrease in protein content, however no genes relating to protein catabolism were differentiated in this experiment (McKenney et al., 1998; Reddy et al., 1995).

There were 4 NADH dehydrogenase subunits significantly differentiated, and although they did not cluster together, they all exhibited down regulation at the 0.26 µg/L exposure group where the most severe physiological and developmental effects were seen. The down regulation of 2 NADH dehydrogenase and cytochrome c seen in cluster 2 (Appendix H2) are essential components of the electron transport chain (ETC). Reduction in their expression is indicative of disruption in energy production at high endosulfan concentrations. Twenty-eight general metabolic genes were also identified as significantly differentiated through microarray analysis of Drosophila exposed to endosulfan (Sharma et al., 2011).

Another pathway involved in energy production is the lactic acid cycle whereby lactate is converted to pyruvate via lactate dehydrogenase (LDH). Very little is known about the functioning of the lactic acid cycle as a form of gluconeogenesis in crustaceans (Henry et al., 1994). Decreased levels of LDH have been seen in several species exposed to endosulfan (Mishra and Shukla, 1997; Reddy et al., 1995; Tripathi and Verma, 2004). Decreased levels of LDH occurred in the 0.05 and 0.26 µg/L exposure groups which support other studies where gluconeogenesis via lactate conversion to pyruvate does not appear to be a primary source of energy. Reddy et al. (1995) speculated that endosulfan could cause effects by interacting with the mitochondrial membrane and thereby decreasing respiratory rates. Interestingly, Tripathi and Verma (2004) noted that decreases in LDH enzyme levels could be reversed by removal of the

animal from the endosulfan contaminated environment. Changes in metabolism seen in this experiment are indications of stress and could further affect survival, development, and immune response of the larvae.

2.4.6 Immune and oxidative stress effects of endosulfan

Crustaceans do not have an adaptive immune system and rely solely on innate immunity in respect to bacteria, viruses and parasites (Johansson and Söderhäll, 1989; Söderhäll and Cerenius, 1998). The crustacean innate immune system is composed of three key components, the cuticle, humoral response and cellular response (James and Xu, 2011). There is little research into the effects of pesticides on the cuticle (James and Xu, 2011); however changes in gene expression of cuticle and moult related genes in this study suggest that endosulfan has significant effects on the cuticle, the first line of defense in the crustacean immune response.

Pesticide exposure elicits a response from both the humoral and cellular pathways. Genes involved in detoxification, lipid peroxidation, oxidative stress and phenoloxidase processes were significantly differentiated in the lobster larvae after endosulfan exposure.

Glutathione S-transferase (GST), an important enzyme in the phase II biotransformation of xenobiotics, was significantly down regulated in the 0.26 µg/L endosulfan exposure group. Black tiger shrimp (Penaeus monodon) exposed to endosulfan could not induce GST activity. However, an increase in GST was observed in toads (Bufo regularis), fruit flies (D. melanogaster) the green snakehead fish (Channa punctatus) and the field crab (Paratelphusa hydrodromus), suggesting that GST

response to endosulfan could be species specific in addition to the response to dose and duration (Dorts et al., 2009; Ezemonye and Tongo, 2010; Pandey et al., 2001; Sharma et al., 2011; Yadwad, 1989). Another closely related enzyme, glutathione peroxidase (GPx), involved in protection against reactive oxygen species (ROS) and lipid peroxidation was down regulated in the 0.26 µg/L endosulfan exposure group. Oxidants are produced naturally through metabolism; however when there is imbalance between production of oxidants and antioxidants, oxidative stress can arise (Sies, 1997). Lipid peroxidation has evolved as a biomarker for oxidative stress because excessive ROS can cause damage to fatty acids and the structural integrity of cell membranes (Dorts et al., 2009). The statistically significant down regulation of GST and GPx and lipid peroxidase in the 0.26 µg/L exposure group could indicate that lobster larvae were unable to protect themselves from the stress at this concentration.

In addition to the glutathione system playing an important role in protecting the organisms against oxidative stress, thioredoxin is equally important as an antioxidant. Environmental stress and exposure to viruses have resulted in an increase in thioredoxin in the Pacific white shrimp (Litopenaeus vannamei) (Garcia-Orozco et al., 2012; Wang et al., 2009). Increased levels of thioredoxin dependent peroxidase occur in the F1 progeny of deltamethrin resistant mosquitoes (Anopheles arabiensis), indicating increased expression levels could be important for pesticide resistance (Müller et al., 2008). Thioredoxins are highly conserved across different species; therefore the diversity of functions could also be true in crustaceans. The up regulation of thioredoxin expression at the endosulfan concentrations of 0.008 to 0.26 µg/L is consistent with other studies where thioredoxin increased under oxidative stress (Svensson and Larsson, 2007; Garcia-Orozco et al., 2012; Wang et al., 2009).

Ferritin is an iron binding protein that converts Fe(II) to Fe(III) eliminating transition metals and reducing ROS (Torti and Torti, 2002; Zhou et al., 2008). The effects of pesticides on ferritin are unknown. An increase in ferritin levels has been observed in Pacific white shrimp (L. vannamei) in response to pH stress and white spot syndrome virus infection (Clavero-Salas et al., 2007; Pan et al., 2005; Zhou et al., 2008). The increase in ferritin expression in lobster larvae at the higher endosulfan concentrations was consistent with an increase in immune response and assisting with oxidative stress. In addition to a direct immune response to environmental contaminants, ferritin is also involved in the induction of phase II detoxification enzymes via oxidative stress response elements (Torti and Torti, 2002).

Most of what is known about the effect of pesticides on the immune system of crustaceans is through studies on humoral and cellular immune responses (James and Xu, 2011). One important immune response is the prophenoloxidase (proPO) cascade that is required for melanization and can be stimulated by both humoral and cellular immune responses (James and Xu, 2011; Johansson and Söderhäll, 1989). Briefly, prophenoloxidase (proPO) is activated by a prophenol activating enzyme (ppA) such as serine protease (Johansson and Söderhäll, 1989). Activated phenoloxidase oxidizes phenol to quinone which subsequently undergoes polymerization forming melanin (Liu et al., 2009). Melanin is an important constituent in the hardening of the exoskeleton and has an active role in the crustacean immune response (Liu et al., 2009; Sritunyalucksana and Söderhäll, 2000). After lobster larvae exposure to endosulfan, two serine proteases exhibited varying expression profiles in all exposure groups with the exception of a significant down regulation in the 0.26 μg/L exposure group. This suggests that the endosulfan exposure was impairing the normal function of the proPO

pathway. Down regulation of the serine proteases is consistent with moult inhibition and down regulation of cuticle related genes in higher endosulfan concentrations. A depressed immune system could also reduce the ability of the lobster larvae to combat infections. Drosophila larvae exposed to endosulfan had a decreased cellular immune response in the ability to encapsulate the eggs of the parasitoid wasp Leptopilina boulardi; this increased the time required to develop from egg to an adult (Delpuech et al., 1996).

Despite ongoing research to unravel the intricacies of the crustacean immune system, most work thus far has focused on mature adults. In larval tiger shrimp (P. monodon) the immune related gene proPO was not expressed during the late nauplii stage; that expression levels increased with maturing developmental stages (Jiravanichpaisal et al., 2007). With an under developed immune system exposure to environmental toxicants could render the larval population susceptible to pathogens and other forms of infection. A current example of such a phenomenon is the decline in the world's amphibian populations, where environmental contaminants are thought to be increasing the susceptibility of amphibians to pathogens such as chytridiomycosis and ranaviral disease, resulting in drastic population declines (Daszak et al., 1999; Hayes et al., 2006 & Rohr et al., 2008).

2.4.7 Gene regulation effects of endosulfan

Exposure of non-target organisms, such as H. americanus larvae to environmental toxicants, can have direct effects on development, metabolism and immunity. There can also be broad ranging effects on gene regulation. After endosulfan

exposure, there were a significant number of genes differentially expressed that were involved in transcription and translation. There were 26 ribosomal proteins identified in cluster 2 (Appendix H2) that showed down or minimal expression in lower exposure groups and an increased expression at 0.05 and 0.26 µg/L groups. Exposure of the water flea (D. magna) to the anti-ecdysteroid fenarimol resulted in down regulation of select ribosomal proteins as a possible energy saving mechanism (Soetaert et al., 2007). Alternatively, the increase seen in this study could indicate up regulation of ribosomal protein gene expression to meet the increasing metabolic and immune response needs as larvae attempt to progress through to metamorphosis. Intermoult H. americanus juvenile males fed heptachlor, an organochlorine, exhibited down regulation of S27E gene in the hepatopancreas (Snyder, 1999) however this was contradictory to what was seen in the current endosulfan exposure study. Interestingly, there have also been examples where increased expression in ribosomal proteins was related to pesticide resistant strains of the mosquito (Culex pipiens) (Wu et al., 2004).

Ubiquitin is found in eukaryotic cells and is involved in DNA transcription, protein degradation and rRNA processing (Korsloot et al., 2004). There were 2 ubiquitin genes identified differentially expressed, ubiquitin conjugating enzyme E2M and ubiquitin/ribosomal protein S30e fusion protein that shared a similar expression profile throughout all endosulfan concentrations in cluster 2 (Appendix H2). Up regulation of the ubiquitin and ribosomal genes observed at 0.05 and 0.26 µg/L exposure groups indicate that endosulfan was impacting transcription and translational processes.

There are 5 families of histone proteins that are involved in the condensation of chromatin and Histone H1 was differentially expressed in the current endosulfan experiment. Histone H1 is a protein that connects the nucleosome to a strand of linker

DNA, and although initially suspected to be involved in global gene repression, studies have indicated that Histone H1 may only repress a small set of genes (Ni et al., 2006; Shen and Gorovsky, 1996; Zlatanova and Van Holde, 1992). Exposure of organisms to environmental stress undoubtedly results in changes in gene expression in order to survive. One area of research that is gaining interest is the change in chromatin structure as a result of environmental stress which is considered a mechanism for epigenetics (Moggs and Orphanides, 2004; Reamon-Buettner et al., 2008). After endosulfan exposure, Histone H1 was up regulated at lower concentrations with decreased expression at higher concentrations. There were significant developmental delays, changes in metabolism and immune response in the 0.05 and 0.26 μg/L exposure groups. Attempts by the larvae to respond to these changes could cause chromatin to becomes less condensed, allowing RNA polymerases to carry out transcription of select genes (Moggs and Orphanides, 2004). Exposure of rat dopaminergic neuronal cells to dieldrin, a persistent chlorinated hydrocarbon, resulted in elevated levels of histone acetylation known to cause chromatin relaxation and increase in transcription (Song et al., 2010).

2.4.8 Microarray and RT-qPCR results comparisons

Microarrays are useful screening tools for ecotoxicology; however expression data should be validated using a complementary gene expression analysis technique such as RT-qPCR. Previously, in RT-qPCR it was common practice to use one housekeeping gene from a standard list of genes that remained stable throughout all organisms, tissues and experimental conditions; however this paradigm is changing (Vandesompele et al., 2002). The microarray data from the endosulfan exposed lobster larvae was useful in

identifying stable candidate reference genes using the geNorm application in qbase^{PLUS} (Biogazelle, Ghent, Belgium). The application was used to apply a statistical algorithm to identify the most stable genes from the endosulfan exposure data set. Using the appropriate reference genes is crucial to the normalization of RT-qPCR data. The use of a single housekeeping gene results in substantial errors in fold changes in 10-25% of samples (Vandesompele et al., 2002).

In the present study, the comparison of microarray and normalized RT-qPCR gene expression resulted in similar expression patterns between both methods; however microarray expression levels were often grossly underestimated. The correlation coefficients varied greatly between GOI from 0.0149 to 0.6416, which is not unusual and occurs elsewhere (Morey et al., 2006). Genes having expression levels below 2 fold, low intensity, location of the primer sequences, and array spot p-values can all affect the degree of correlation between the two methods (Beckman et al., 2004; Etienne et al., 2004; Morey et al., 2006). To reduce variation between methods, microarray features used in the extraction of expression data were filtered through stringent flagging parameters, primers were specifically designed around the EST and or gene sequence corresponding to the oligonucleotide probe sequence when possible. Only good quality RNA was used for both methods. Correlations are often not reported in many studies, however as microarray technology and analysis continue to develop, there is also a need for more research to ensure consistency. The reporting methods for both microarray and RT-qPCR follow MIAME and MIQE standards respectively. Conforming to these reporting guidelines is important to ensure that experiments are reproducible and important experimental information is provided to the reader (Brazma et al., 2001 and Bustin et al., 2009).

2.5 Conclusion

This study provides novel insights into the toxicological, developmental and molecular effects of endosulfan on H. americanus larval metamorphosis. Endosulfan is considered to be a persistent organic pollutant that can remain in the environment long after its use. Developmental delays, deformities, changes in metabolism and reproductive effects have been seen in other species after endosulfan exposure (Brunelli et al., 2009; Reddy et al., 1995; Sharma et al., 2011; Velasco-Santamaría et al., 2011). Metamorphosis is a critical developmental milestone and changes in the environment and exposure to pollutants can severely affect the progression of metamorphosis (Capuzzo et al., 1984). The mechanism by which endosulfan affects development is unknown, however microarray analysis has presented some interesting possibilities.

Changes in gene expression related to cuticle formation such as chitinases, cuticular proteins and dumpy, correlate well with developmental delays observed throughout the larval lobster exposure experiment. Several genes, FAO-MeT, EIP-75 and FTZ-F1, involved in ecdysteroid signaling during lobster larval moult and metamorphosis were also differentially expressed. Studies on Drosophila specifically illustrate the importance of EIP-75 and FTZ-F1 for normal moult and cuticle development (Bialecki et al., 2002; Dubrovsky et al., 2011; Riddiford et al., 2003; Sullivan and Thummel, 2003).

Changes in moult may be a result of general stress response and changes in energy allocation (Mazurová et al., 2008). An increase in metabolic genes such as AMP dependent CoA ligase, flavoprotein ubiquinone and mitochondrial trifunctional protein

suggest that up regulation of beta oxidation is required to meet energy requirements in larval lobster exposed to endosulfan. There was however a decreased expression of NADH dehydrogenase that would suggest negative effects of endosulfan on the ETC. The simultaneous down regulation of lactate dehydrogenase (LDH) in endosulfan exposed larvae suggests that anaerobic metabolism cannot assist in the production of ATP.

Gene expression analysis also identified several immune and oxidative stress related genes that were significantly differentiated at higher endosulfan concentrations. The serine protease ppA and glutathione peroxidase were up regulated at lower endosulfan concentrations, where perhaps they could afford to expend the energy to increase their immune and oxidative stress response system; however at higher endosulfan concentrations both genes exhibited a down regulation. On the contrary, thioredoxin and ferritin were up regulated at higher endosulfan concentrations, indicating that not all components of the immune system were compromised.

Finally, endosulfan appeared to have considerable effects on overall lobster larval gene regulation. Twenty-six ribosomal proteins were clustered together, often having increased expression at the higher endosulfan concentrations. Although this is contradictory to Soetart's (2007) study that found a down regulation of select ribosomal proteins, the increase in expression was needed for an enhanced metabolic or immune response in larval lobster.

This study demonstrated that analysis of global gene expression using a lobster specific oligonucleotide microarray was useful in identifying genes involved in developmental processes, metabolism, immune response and gene regulation that were significantly affected by endosulfan exposure. While having only ~40% annotation, 707

genes were identified as being statistically differentiated, many with interesting biological and molecular functions. Despite a recent ban on the use of endosulfan in the United States and Canada, endosulfan is a known POP and will be present in the environment after its discontinuation. This study was integral in identifying the effects of endosulfan on H. americanus and developing microarray technology for future environmental monitoring practices.

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3.0 GENERAL DISCUSSION

3.1 Summary of endosulfan exposure and gene expression analysis

The American lobster, H. americanus, is an important economic species in Atlantic Canada and the survival and health of lobster larvae is essential for maintaining viable lobster populations. Lobster larvae are found in the pelagic zone; they are susceptible to environmental conditions such as changes in temperature, salinity, photoperiod and exposure to environmental pollutants (Ennis, 1995). Agricultural pesticides in PEI are typically applied during the spring and summer months, which coincides with the presence of lobster larvae in the water column. The effects of endosulfan on metamorphosis has been studied in tadpoles (Brunelli et al., 2009) fruit flies (Sharma et al., 2011) and water fleas (Palma et al., 2008) and shown to cause developmental delays and deformities in tadpoles and fruit flies in addition to changes in the moult cycle of fruit flies and water fleas. Endosulfan exposure to lobster larvae transitioning through metamorphosis resulted in developmental delays and changes in moult regulation, as seen in other species. Genomic analysis also highlighted changes in gene expression affecting other developmental processes, metabolism, immune and oxidative stress response and gene regulation.

Traditional toxicology endpoints on model organisms are identifying LC_{50} concentrations, monitoring developmental deformities and screening for biological markers, such as CYP 450 and GST that are indicative of stress and detoxification. Microarrays are useful high throughput tools that allow for the monitoring of thousands of genes simultaneously and from which new gene biomarkers may be discovered. Genes of interest that were significantly differentiated after endosulfan exposure were

identified in this study, however further studies are required before these genes could be classified as endosulfan biomarkers.

For many environmental contaminants, the lethal mode of action (MOA) may not be known and often the sublethal effects are not fully characterized. Microarray gene expression analysis of lobster larvae exposed to chronic endosulfan concentrations was useful in identifying genes involved in various pathways and processes that were negatively affected. Chronic exposure to endosulfan had significant effects on development, moulting, metabolism, immunity, and gene regulation.

H. americanus is not a standard research organism, and with only ~40% of the genes on the array were functionally annotated, therefore there was a considerable amount of data that could not be analyzed. Despite lack of annotation, the k-means cluster analysis was useful to identify genes with similar expression patterns that could be involved in similar pathways or processes. Regardless, 707 genes showed differentially significant responses to endosulfan exposure including genes involved in development, metabolism, immune and oxidative stress response and gene regulation. Nine functionally annotated genes of interest (GOI) were selected for RT-qPCR validation of the microarray results. RT-qPCR is known to be a more sensitive detection method and was useful in determining if expression levels seen in the microarray analysis were accurate. Consequently, RT-qPCR was able to identify a similar expression pattern as the microarray; however the level of expression was often greater than the microarray.

3.2 Future directions

The analysis of gene expression using lobster specific microarray completed in the present study was useful in identifying pathways and processes affected by endosulfan exposure that warrants further study. To build on this research the following areas would be worthwhile to investigate:

- The establishment of baseline information on normal gene expression during metamorphosis and throughout the moult cycle. A limitation of the current study was that temporal gene expression studies ("age in stage") on larval moult and metamorphosis under defined experimental conditions were not available. This information would allow for greater interpretation of pesticide exposure data.

- Collecting gene expression information from an acute (96 h) endosulfan exposure would allow for comparisons with chronic exposure data from the current study. This would be useful in determining if similar gene expression patterns are present or if an acute exposure elicits a completely different response at the gene expression level.

- Performing pesticide exposures on H. americanus of other organochlorines such as lindane and dieldrin would determine if there are any similarities in the gene expression response within a family of pesticides. This would help focus the search for identifying unique biomarkers for specific pesticides.

- Although biologically interesting genes were identified in this study, future work could focus on the effects of endosulfan on signaling pathways in the lobster larvae. This would provide further support for identifying biomarkers or contaminant specific gene expression profiles.

- One of the long term goals for our laboratory is to use microarray technology as a screening tool for environmental contaminant samples. For microarray experiments to become a valid test method, considerable work must be done to create a standardized method for performing the experiment and analyzing the results. The development of MIAME guidelines are a great start, however research in this area must continue before microarray results can be considered a reproducible and reliable test method.

3.3 Concluding remarks

There is growing concern throughout the world about the pollutants being emitted into the environment and the effects they have on species that live within those environments. The study of contaminants now encompasses different fields of research with an increasing emphasis on the "omics" such as genomics, proteomics and metabolomics. If species populations are to survive and flourish in the future, there is an urgent need to understand how pollutants are affecting the environment in order to prevent further damage and to rectify damage that has already been done. Microarrays provide high throughput technology that allow for the extraction of large quantities of data that can assist in the identification of pollutant modes of action and will hopefully be used for environmental monitoring in the future.

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APPENDICES

Appendix A: Sample collection

Lobster larvae, Homarus americanus, were cultured at Coastal Zone Research Institute (CZRI)-Homarus Inc. in Shippagan, New Brunswick Canada. Berried female lobsters were held until hatching occurred at which time the new emerged larvae were collected. On the day of the hatch, approximately 3000 stage I larvae were obtained and transported back to Environment Canada Toxicology Laboratory in Moncton, New Brunswick (ALET). At the CZRI facility, the larvae were held in ambient water flow through holding tanks until pick up. The water quality and parameters were not available for publication; however the larvae were collected within a few hours of hatching and transported back to the Environment Canada Toxicology Laboratory (ALET) where detailed culture methods were implemented (Methods and Materials).

Appendix B: TRIzol®/RNeasy® RNA extraction with DNase clean up

*Note: The protocol was taken from an uncontrolled copy of Environment Canada SOP-TOX85 and was developed at Environment Canada (ALET) in Moncton.

Step 1 Remove 6-18 homogenized samples from the -80 ºC and let thaw at room temperature.

Step 2 Pipette 200 µl of chloroform into a 1.5 ml RNase free tube for each sample in the fumehood.

Step 3 Once the homogenate has thawed, it is poured into tubes containing chloroform and carefully inverted 15 times.

Step 4 Let the sample sit for 2-3 min at room temperature

Step 5 The samples are loaded into the refrigerated centrifuge and are spun at 8000 × g for 15 min at 4 ºC.

Step 6 The supernatant is carefully transferred into a new 1.5 ml RNase free tube. It is important that no debris from the protein layer is accidentally transferred, as this will contaminate the RNA extract.

Step 7 An equal amount of 70% ethanol is pipetted in with the supernatant and gently mixed using the pipette.

Step 8 An aliquot of < 700 µl of the supernatant is pipetted into an RNeasy column with collection tube and spun at 8000 × g for 30 s at room temperature and the flow-through discarded.

Step 9 If the volume of the supernatant was more than 700 µl, repeat 6-8 until all the supernatant has been filtered.

Step 10 350 µl of RW1 buffer solution (Qiagen RNA-Mini Prep Kit) is pipetted onto the RNeasy column and spun at 8000 × g for 30 s at room temperature and the flow-through discarded

Step 11 10 µl of DNase I stock solution* (Kit) and 70 µl of RDD buffer solution (Kit) are pipetted onto the RNeasy column and allowed to sit at 20-30 ºC for 15 min. DNase (lyophilized powder) can be pre-prepared ahead of time and kept in the -20 ºC freezer until use. They can be pre-aliquot in 0.2 ml tubes containing 130 µl of DNase solution (enough to be used with 12 samples). Only on the day of use, the DNase solution can be mixed with the RDD buffer solution.

*Note: DNase I stock solution is made by adding 550 µl of nuclease free water to the lyophilized DNase I stock.

Step 12 After DNase treatment, 350 µl of RW1 buffer solution (Qiagen RNA-Mini Prep Kit) is pipetted onto the RNeasy column and spun at 8000 × g for 30 s at room temperature and the flow-through discarded.

Step 13 500 µl of RPE buffer* (Qiagen RNA-Mini Prep Kit) containing 100% ethanol was pipetted onto the RNeasy column and spun at 8000 × g for 30 s at room temperature and the flow-through discarded.

*Note: The RPE buffer is a concentrate and 220 µl of 100% ethanol was added to the buffer before use.

Step 14 500 µl of RPE buffer is pipetted onto the RNeasy column and spun at 8000 × g for 2 min at room temperature and the flow-through discarded.

Step 15 The column is transferred to a new collection tube and spun dry at 8000 x g for 1-2 min at room temperature.

Step 16 The column is transferred to a new 1.5 ml RNase free tube and 30 µl of warm nuclease free water is added to the RNeasy column. Let samples sit at room temperature for 1-2 min before being spun at 8000 × g for 1 min at room temperature. Be sure to KEEP supernatant. The nuclease free water can be kept warm at 60 ºC on a hot plate until use. The heated water will help to extract RNA more efficiently.

Step 17 Step16 is repeated to bring total extracted volume to 60 µl.

Step 18 2 µl of each sample is pipetted into two 0.2 µl RNase free tubes containing 18 µl of nuclease free water for RNA quantity and quality analysis.

Step 19 2 µl of RNA inhibitor is added to each eluded RNA stock solution and samples are stored in at -80 ºC until further use.

Appendix C: Assessing the quantity of total RNA using the Nanodrop spectrophotometer

*Note: The protocol was taken from an uncontrolled copy of Environment Canada SOP-TOX86 and was developed at Environment Canada (ALET) in Moncton.

Step 1 All procedures must be done on a DNA and RNA free surface. RNaway spray/wipes were used to wipe down surfaces and tube racks.

Step 2 Pre-aliquot total RNA extract samples (20 µl-1:10 dilution) are removed from the -80 ºC freezer and thawed at room temperature.

Step 3 Wipe down the 8 wells of the nanodrop using a Kimwipe and pipette 2 µl of nuclease free water onto each of the 8 wells and close the lid. The nanodrop will automatically initialize and blank the wells.

Step 4 Open the ND-8000 software and select "nucleic acid" option on the menu.

Step 5 Select "RNA-40" from the drop down menu, activate all the wells and blank again. Once complete, wipe down the wells and lid with a Kimwipe.

Step 6 Be sure that all the wells going to be used are activated on the software and that the "recording" button is on.

Step 7 Pipette 1.5 µl of sample onto the wells and close the lid. On the software, select "measure" and the results will be on the screen.

Step 8 Repeat step 6 until all the samples have been quantified.

Step 9 On the software, click "show report" that can be saved as an excel report if desired in a file folder. The RNA concentration showed in the report is measured in a 1:10 diluted sample. Therefore, all the measurements will need to be multiplied by 10 to provide the real concentration of RNA in the samples.

Step 10 The nanodrop needs to be cleaned after every run. Five microlitres of nuclease free water was pipetted onto each well, lid closed and left for 5 min and then wiped down with Kimwipes.

Appendix D: Assessing the quality of extracted total RNA using Bio-Rad experion microfluid chip

*Note: The protocol was taken from an uncontrolled copy of Environment Canada SOP-TOX87 and was developed at Environment Canada (ALET) in Moncton.

**Note: Upon arrival of the RNA ladder, 1 µl of the RNA ladder was added to 6 µl of gel loading buffer in 0.2 ml tubes and held at -80 ºC until use. The remainder of the Experion ™ RNA StdSens Analysis Kit was held at 4 ºC.

Step 1 All procedures must be done on DNA and RNA free surfaces. RNaway spray/wipes were used to wipe down surfaces and tube racks.

Step 2 The Experion electrophoresis machine needs to be cleaned prior to use. Place 800 µl of nuclease free water into two cleaning chips labelled "H_20" and 800 µl of cleaning solution onto the chip labelled "cleaner".

Step 3 Place the cleaning chip containing the cleaner solution into the electrophoresis chamber's electrode, close the lid and let soak for 5 min.

Step 4 Repeat step 3 but using the two cleaning chips labelled "H_20", let sit for 5 min and then let air dry for another 5 min.

Step 5 Pre-aliquot total RNA extract samples and RNA ladder are removed from the -80 ºC and thawed at room temperature.

Step 6 Twelve aliquots of 6 µl of gel loading buffer are pipetted into 0.2 ml tubes. Once the samples have thawed, briefly spin down in the mini-spin centrifuge.

Step 7 1 µl of sample is pipetted into each 0.2 ml tube containing the gel loading buffer. If less than 12 samples add 1 µl of water to the extra gel loading buffer tubes.

Step 8 1 µl of ladder is pipetted into 0.2ml tube containing the gel loading buffer.

Step 9 All samples and the ladder are placed in the thermocycler and the protocol started.

Step 10 A gel stain solution is prepared by adding 1 µl of blue stain solution* into 65 µl of filtered gel**.

***Note: Keep the gel stain solution out of light when not in use.

****Note: If this is the first time using the gel stain solution, 600 µl of RNA gel is pipetted into a spin filter tube and centrifuged 1,500 × g for 10 min prior to use. The filtered gel is good for a month after which time is should be re-filtered.

Step 11 Prior to the chip being used, it must be primed. Nine microlitres of the gel stain solution is pipetted into the chip well labelled "GS" (white) and the chip is placed in the priming station. The priming station is set to "1" and pressure "B" and started.

Step 12 When the priming is complete, the chip is removed and 9 µl of the gel stain solution is pipetted into the chip well labelled "GS" (black) and 9 µl of filtered gel solution is pipetted into the well labelled "G".

Step 13 The samples and the RNA ladder are removed from the thermocycler and vortexed.

Step 14 Six microlitres of each sample and RNA ladder are pipetted into the corresponding well on the gel chip. Once all the wells are full, the chip is placed into the experion electrophoresis chamber and the lid closed.

Step 15 Turn on the electrophoresis machine and open the experion software and select "new run", "eukaryote total RNA StdSens" from the pull down menu.

Step 16 When the run is complete, a gel and graph are created and can be saved as a PDF format.

Appendix E: Hybridization buffer solutions

Channel #1: 5X SSC, 0.01% SDS, 0.2% Bovine Serum Albumin (BSA)
-250 ml of 20X SSC
-1 ml of 10% SDS
-20 ml of 10% BSA
-729 ml Milli-Q Water

Channel #2: 2X SSC, 0.2% SDS
-100 ml of 20X SSC
-20 ml of 10% SDS
-880 ml of Milli-Q Water

Channel #3: 0.2X SSC
-10 ml of 20X SSC
-990 ml of Milli-Q Water

Channel #4: 5X SSC
-250 ml of 20X SSC
-750 ml of Milli-Q Water

Channel #5: 0.2X SSC, 0.2% SDS
-10 ml of 20X SSC
-20 ml of 10% SDS
-970 ml of Milli-Q Water

Appendix F: Tecan HS4800Pro hybridization protocol

*Note: It is recommended in the Tecan HS480Pro manual that a pre-hybridization be run before every hybridization using blank slides. The pre-hybridization is also run when the machine has not been used for several days or weeks. If the machine was going to be used for several consecutive days, the pre-hybridization protocol was not run.

**Note: Prior to pre-hybridization or hybridization, each channel is primed for 30 s with the corresponding hybridization buffer. Buffers containing SDS are placed on the heating block and are heated throughout the pre-hybridization/hybridization protocol. If the module does not contain 4 microarray slides, blank slides are placed in free chambers.

Pre-Hybridization Protocol

1. Wash: Temp 23 ºC; Channel 3; Run: 1, Wash time 30 s, Soak time 30 s

2. Wash: Temp 23 ºC; Channel 2; Run: 1, Wash time 30 s, Soak time 30 s

3. Wash: Temp 37 ºC; Channel 2; Run: 1, Wash time 30 s, Soak time 30 s

4. Hybridization: Temp 46 ºC; Agitation Frequency: Medium, High Viscosity Mode: Yes, Time: 16 h

5. Wash: Temp 23 ºC; Channel 2; Run: 1, Wash time 30 s, Soak time 0 s

6. Wash: Temp 37 ºC; Channel 3; Run: 1, Wash time 20 s, Soak time 0 s

7. Slide drying: Temp30 ºC, Time: 2 min, Final Manifold Cleaning: No, Channel: No

8. Place all channels in Milli-Q water and start the rinse and drying cycle

Hybridization Protocol

1. Hybridization: Temp 65ºC, Agitation Frequency: No, High Viscosity Mode: No, Time: 10min

2. Wash: Temp 65 ºC; Channel 1; Run: 1, Wash time 20 s, Soak time 0 s

3. Wash: Temp 50 ºC; Channel 1; Run: 1, Wash time 20 s, Soak time 0 s

4. Hybridization: Temp 50 ºC, Agitation Frequency: No, High Viscosity Mode: Yes, Time: 13 min

5. Wash: Temp 50 ºC, Channel 1, Run: 1, Wash time: 20 s, Soak Time: 0 s

6. Hybridization: Temp 48 ºC, Agitation Frequency: High, High Viscosity Mode: Yes, Time: 13 min

7. Wash: Temp 45 ºC, Channel 1, Run: 1, Wash time: 1 min, Soak Time: 0 s

8. Wash: Temp 48 ºC, Channel 4, Run: 1, Wash time: 1min, Soak Time: 1min

9. Sample Injection: Temp 48 ºC, Agitation: Yes, BCR: No

10. Hybridization: Temp 48 ºC, Agitation Frequency: High, High Viscosity Mode: Yes, Time: 16 h

11. Wash: Temp 41 ºC, Channel 2, Run: 1, Wash time: 1 min, Soak Time: 0 s

12. Hybridization: Temp 48 ºC, Agitation Frequency: Medium, High Viscosity Mode: No, Time: 2 min

13. Wash: Temp 30 ºC, Channel 2, Run: 1, Wash time: 1 min, Soak Time: 0 s

14. Wash: Temp 30 ºC, Channel 5, Run: 1, Wash time: 1 min, Soak Time: 0 s

15. Hybridization: Temp 30 ºC, Agitation Frequency: Medium, High Viscosity Mode: No, Time: 2 min

16. Wash: Temp 23 ºC, Channel 5, Run: 1, Wash time: 1 min, Soak Time: 0 s

17. Wash: Temp 23 ºC, Channel 3, Run: 1, Wash time: 1 min, Soak Time: 0 s

18. Hybridization: Temp 23 ºC, Agitation Frequency: Medium, High Viscosity Mode: No, Time: 2 min

19. Wash: Temp 23 ºC, Channel 3, Run: 1, Wash time: 1 min, Soak Time: 0 s

20. Slide Drying: Temp 30 ºC, Time: 3 min, Final Manifold Cleaning: No, Channel: No

21. Remove microarray slides, replace with blank slides and begin the rinsing protocol. Remove all channels from buffer solutions, place in Milli-Q water and start the rinsing and drying protocol.

Appendix G: Primer sequences for RT-qPCR validation

A three step RT-qPCR protocol was used with SYBR GreenER qPCR master mix. The RT-qPCR cycle was 50 ºC for 2 min, 95 ºC for 2 min followed by 40 cycles of 95 ºC for 7 sec, 20 sec at the primer specific annealing temperature, 72 ºC for 20 sec after which the plate was read. A melt curve was also run from 65-90 ºC with each temperature being held for 2 sec and a reading was taken at 0.5 ºC intervals. The probe ID, name, melting temperature, amplicon length (bp), efficiency and identification of the probe sequence within the amplicon region are listed for all genes.

Probe ID	Name	°C	Amplicon (bp)	Efficiency (%)	Probe Sequence	Primer	Sequence
HAC00663	Glutathione s-transferase	61.4	169	101.80	Yes	Forward Reverse	TGCGATCATGCGTTACATTGACG AAGAGCATCTAGGTACCGCTGCT
HAZ04177_1	NADH dehydrogenase	57.5	166	101.6	Yes	Forward Reverse	TTTGTTAGGTAGGCTTCGGGCTGT TAAAGCTAACGGCAACGTGACCCA
HAZ16336_1	Histone-H1	59.2	107	98.35	Yes	Forward Reverse	CTCCGGCTCCTTCAGAATCAACAA TCTTAGCAACGGGCTTCTTGACGA
HAZ11503_1	Ecdysone nuclear receptor FTZ-F1	66.1	103	102.21	Yes	Forward Reverse	TGGAATCTGTTCGTGCTGACCGTA TTGTCGCTGTCGGAGCATCTGTAA
HAZ04719_1	Ferritin 1 heavy chain homologue	59.2	196	100.69	No	Forward Reverse	TCTGCAACAGAGGAGCGAAGTCAT TAAGGGCTTGCTCTAAGGCTGCTT
HAC01818	Thioredoxin	64.0	157	102.43	Yes	Forward Reverse	TTAGGGCTGGACACCTTTCACCTT TGTGCATTAAGGTCAGCCTCCATC
HAZ15352_1	Cuticle Protein	64.0	177	102.43	No	Forward Reverse	TCCTGGTAGTTCTGGCTTTCGCTT AGTACTGGCCAACAACACTACCGT

HAC03276	Farnesoic acid o-methyltransferase	61.4	82	101.13	Yes	Forward Reverse	GGCCACAGGCAAGTGGAAATTTGA ATATGTGTGGCGTGTGTGTGTGAC
HAZ10698_1	Ecdysone inducible protein E75	62.5	97	93.3	No	Forward Reverse	CACCAAGAACCAACAGTGCTCCAT ATCTCTGGACATGCCGACAGCAAT
HAZ11665_1	Brefeldin A (Bref)	61.4	111	106.2	No	Forward Reverse	GCTTTGCCAATGCCCAAGAGTGAT AAGGGCTGACACCACAATCTTTGC
HAZ11301_1	Microtubule associated protein (R301)	57.0	108	101.4	No	Forward Reverse	CAAGATCTTGCAGTATGCCTT TGAACCACTGAAGGAATTCA
HAZ08411_1	Riboflavin kinase (R411)	65.0	148	100.4	Partially	Forward Reverse	ACGGATCTGAACTGAAGGTGGTGA AGTGCAGATGTTGGGCCTGTAAGA
HAZ01565	Hypothetical protein LOC618343 (Hyploc)	62.5	140	89.0	No	Forward Reverse	ACATGGCAGTGGAAGACTCAAGGA AAAGGAAACTGCGAACACTGCTGG
HAZ00289	Conserved hypothetical protein (CHP)	66.5	90	92.0	No	Forward Reverse	TCAAGCCTGAAGCTGGGATATGCT AAACACATGGGTTGGATGGCACAG

Appendix H: Statistically significant differentially expressed genes

Genes were determined to be significantly differentiated if there was 1.5-fold difference, were statistically significant after 1 way-ANOVA and 100 permutations (p-value < 0.05). Genes were further analyzed with k-means cluster analysis and assigned to 4 unique clusters (H1-4). The probe ID, accession number, gene name, species and p-value are listed for each gene.

H1: Statistically significant differentially expressed genes included in K-means cluster 1 (n=95).

Probe ID	EST Accession #	Protein Name	Source	p-value
Development				
HAZ04898_1	DV772458	Angio-associated migratory protein	Mus musculus	0.02
HAZ12715_1	FD699176	Crustacyanin-A2 subunit	N/A	0.04
Metabolism				
HAZ14861_1	FD483172	Bicoid-interacting protein 3 CG8276-PA, isoform A	Drosophila melanogaster	0.05
HAZ04470_1	FE841409	Cathepsin A isoform b	Mus musculus	0.04
HAZ10158_1	FD584995	Hypothetical protein AaeL_AAEL004278	Aedes aegypti	0.05
HAZ13371_1	FC071245	Mitogen activated protein kinase kinase kinase 5, mapkkk5, mekk5	Nasonia vitripennis	0.05
HAZ10827_1	EX471437	Protein arginine N-methyltransferase-like protein	Arabidopsis thaliana	0.01
HAZ08417_1	EH116295	Salivary secreted ribonuclease	Nasonia vitripennis	0.05
HAZ11043_1	EX486533	serpentine CG32209-PB	Drosophila melanogaster	0.01
Immune & Oxidative Stress Response				
HAZ09738_1	EX471060	AGAP003014-PA	Tribolium castaneum	0.02
Transcription/Translation				

HAZ09812_1	FE043854	Casein kinase 2, beta subunit isoform 2	Macaca mulatta	0.03
HAZ10181_1	EW703133	Dicer-1	Penaeus monodon	0.04
HAZ07254_1	FE535083	DNA replication complex GINS protein PSF1	Danio rerio	0.03
HAZ06992_1	EH401770	Ornithine decarboxylase	Nasonia vitripennis	0.05
HAZ09125_1	EX486885	Splicing factor 1 CG5836-PA	Apis mellifera	0.04
HAZ15706_1	FD585293	Splicing factor arginine\serine-rich 6	Bombyx mori	0.02
HAZ11959_1	EX568212	28S ribosomal protein S5	Tribolium castaneum	0.02
HAZ13243_1	EY291232	Eukaryotic translation elongation factor 1 beta	Drosophila yakuba	0.04
HAZ05041_1	DV772659	Eukaryotic translation initiation factor 3, subunit M	Homo sapiens	0.03
HAZ07467_1	EH034750	Nocturnin CG31299-PE	Tribolium castaneum	0.03
Other Genes				
Annotated: 18 Unannotated: 56				P <0.05

H2: Statistically significant differentially expressed genes included in K-means cluster 2 (n=253).

Probe ID	EST Accession #	Protein Name	Source	p-value
Development				
HAZ15038_1	FD483499	Knickkopf CG6217-PA, partial	Tribolium castaneum	0.05
HAC01819	EY116762	Myosin light chain	Tribolium castaneum	0.04
HAC03187	EX471288	Tetracycline resistance	Culex quinquefasciatus	0.01
HAC03488	CN951905	Wingless-type MMTV integration site family, member 2B isoform WNT-2B1	Homo sapiens	0.01

Metabolism				
HAZ14899_1	FD483237	AMP dependent coa ligase	Culex quinquefasciatus	0.02
HAZ10802_1	EX471394	Choline kinase R2	Rattus norvegicus	0.03
HAZ07645_1	EH035012	Electron transfer flavoprotein-ubiquinone oxidoreductase	Ornithorhynchus anatinus	0.04
HAZ11463_1	FF277365	GJ24648	Drosophila virilis	0.04
HAZ07246_1	FE659872	Mitochondrial trifunctional protein beta subunit	Danio rerio	0.011
HAC02625	CN950784	NADH-ubiquinone oxidoreductase 42 kda subunit	Tribolium castaneum	0.03
HAZ12955_1	EY290726	Ubiquitin-conjugating enzyme E2M	Apis mellifera	0.01
HAZ04560_1	DV771969	UDP-N-acetylglucosamine--dolichyl-phosphate N-acetylglucosaminephosphotransferase (N-acetylglucosamine-1-phosphate transferase) (GlcNAc-1-P transferase)	Gallus gallus	0.01
HAZ04032_1	DV774338	Glutamine synthetase (Glutamate--ammonia ligase)	N/A	0.01
Immune & Oxidative Stress Response				
HAZ04719_1	DV772251	Ferritin 1 heavy chain homologue CG2216-PE, isoform E	Apis mellifera	0.01
HAZ_CL20_1	EX568422	Anti lipopolysaccharide factor 2	Homarus americanus	0.05
HAZ06079_1	DV774220	Anti-lipopolysaccharide factor like protein	Marsupenaeus japonicus	0.04
HAC03200	EH116796	Omega class glutathione S-transferase	Crassostrea gigas	0.01
HAC01818	FE659629	Thioredoxin domain-containing protein 16 precursor	N/A	0.03
HAC03425	CN951822	Thymidylate synthetase isoform 3	Macaca mulatta	0.01
Transcription/Translation				

HAC03657	FE659818	60S acidic ribosomal protein P2	Aspergillus clavatus	0.01
HAC00603	FE535117	40S ribosomal protein S14	N/A	0.01
HAZ14954_1	FD699856	40S ribosomal protein S27	N/A	0.01
HAC00799	FE841504	60S ribosomal protein L38	Brugia malayi	0.03
HAZ05158_1	FD468078	Elongation factor 1 beta	Diaphorina citri	0.02
HAC00072	FE841157	Elongation factor 1-alpha	Upogebia major	0.01
HAZ08992_1	EH117182	Endonuclease\ reverse transcriptase	Strongylocentrotus purpuratus	0.05
HAZ_CL12_1	CN949905	Hypothetical protein	Ruminococcus gnavus	0.02
HAZ13310_1	FD483812	Pontin	Tribolium castaneum	0.02
HAZ04654_1	FE043814	Ribosomal protein L10	Callinectes sapidus	0.01
HAZ04101_1	DV771358	RNA-dependent helicase p72 (DEAD-box protein p72) (DEAD-box protein 17) isoform 4	Canis familiaris	0.01
HAZ15407_1	FE044263	TAF12 RNA polymerase II, TATA box binding protein	Xenopus laevis	0.01
HAZ18049_1	FF277227	DNA repair protein	Tribolium castaneum	0.01
HAC01671	EH035483	Eukaryotic translation initiation factor 3	Ixodes scapularis	0.02
HAC00812	FE535088	Ribosomal protein L13	Danio rerio	0.01
HAZ_CL33_1	FF278016	Ribosomal protein L23	Haemaphysalis qinghaiensis	0.01
HAZ04854_1	EX487563	Ribosomal protein L3 CG4863-PA, isoform A	Apis mellifera	0.03
HAZ06772_1	EX487234	Ribosomal protein L30	Gallus gallus	0.01
HAZ06901_1	FD699124	Ribosomal protein L37a CG9091-PA	Drosophila melanogaster	0.01
HAC03361	FE535450	Ribosomal protein L7e	Nasonia vitripennis	0.01
HAZ13778_1	FC555910	Ribosomal protein S10	Sipunculus nudus	0.02
HAZ07525_1	EX827155	Ribosomal protein S24	Marsupenaeus japonicus	0.01
HAZ07378_1	FD467527	Ribosomal protein S5 isoform 1	Apis mellifera	0.01
HAZ02651_1	DV771279	Translation elongation factor 2	Tribolium castaneum	0.01
HAC01417	FE043584	DNA-damage inducible protein	Tribolium castaneum	0.01
HAC00209	CN852665	Ribosomal protein L8	Litopenaeus vannamei	0.01

HAC00618	EH035698	60S ribosomal protein L7A	Ixodes pacificus	0.02
HAC01352	CN853932	Ribosomal protein L12	Acyrthosiphon pisum	0.01
HAC01432	EX487429	Ribosomal protein L6e	Carabus granulatus	0.01
HAC01743	CN854379	Ribosomal protein L21	Mus musculus	0.01
HAC02151	CN950184	Ribosomal protein L9 CG6141-PA, isoform A	Drosophila melanogaster	0.01
HAZ06932_1	FC556272	Ribosomal protein S25	Ixodes scapularis	0.01
Other Genes				
Annotated: 46 Unannotated: 156				p<0.05

H3: Statistically significant differentially expressed genes included in K-means cluster 3 (n=104).

Probe ID	EST Accession #	Protein Name	Source	p-value
Development				
HAC03386	CN951775	Chitinase	Scylla serrata	0.02
HAZ_CL17_1	FE660091	Cuticular protein 111, RR-3 family	Acyrthosiphon pisum	0.04
HAZ14720_1	FD468139	Cuticular protein 61, RR-1 family	Anopheles gambiae	0.01
HAZ07865_1	FD468139	DEAD box ATP-dependent RNA helicase	Culex quinquefasciatus	0.01
HAZ09733_1	EV782032	Dumpy CG33196-PB	Drosophila melanogaster	0.01
HAC03220	FD467619	Glucosamine-6-phosphate isomerase	Culex quinquefasciatus	0.01
HAZ09532_1	EV781709	Inhibitor of apoptosis 3	Choristoneura fumiferana	0.01
HAC02644	EX487025	Myosin 3 light chain	Lonomia obliqua	0.01
HAZ13885_1	FC556115	Zinc knuckle domain protein	Culex quinquefasciatus	0.03
Metabolism				
HAZ18290_1	FF277703	4-hydroxyphenylpyruvate dioxygenase	Nasonia vitripennis	0.01
HAC02265	FE043973	ATP synthase B chain, mitochondrial	Culex quinquefasciatus	0.02

HAZ07991_1	EH035515	Histidine decarboxylase CG3454-PA	Apis mellifera	0.01
HAZ03892_1	DV771087	L-3-hydroxyacyl-Coenzyme A dehydrogenase	Xenopus tropicalis	0.01
HAZ04050_1	DV774077	NADH dehydrogenase subunit 3	Geothelphusa dehaani	0.01
HAZ08520_1	EH401619	Adenylosuccinate synthetase	Apis mellifera	0.04
HAZ07445_1	EH034715	Cytochrome c	Marsupenaeus japonicus	0.02
HAZ11523_1	EX487346	Diuretic hormone 31 CG13094-PC	Acyrthosiphon pisum	0.01
HAZ04642_1	DV773863	Mitochondrial ATP synthase F chain	Culex quinquefasciatus	0.01
HAZ04177_1	FD483371	NADH dehydrogenase subunit 1	Litopenaeus vannamei	0.01
HAC01420	CN854011	Trehalose transporter AgTRET1	Anopheles gambiae	0.05
HAC01359	CN951051	Xanthine dehydrogenase	Drosophila mercatorum	0.04
Immune & Stress Response				
HAZ06191_1	DV774376	Serine proteinase	Tenebrio molitor	0.01
HAZ13464_1	FC071413	Mitochondrial manganese superoxide dismutase	Macrobrachium rosenbergii	0.02
HAZ06854_1	EG948772	Hypoxia-inducible factor 1 alpha	Palaemonetes pugio	0.01
Transcription/Translation				
HAC00270	CN852733	Apoptotic chromatin condensation inducer	Danio rerio	0.03
HAC01290	CN853855	Chromodomain helicase DNA binding protein	Acyrthosiphon pisum	0.01
HAZ09438_1	FE841312	Geminin L	Xenopus laevis	0.05
HAZ08383_1	FF278126	Reverse transcriptase	Anguilla japonica	0.01
HAZ13145_1	EY291074	Ribosomal protein L30	Gallus gallus	0.04
Other Genes				
Annotated: 16 Unannotated: 59				$p<0.05$

H4: Statistically significant differentially expressed genes included in K-means cluster 4 (n=255).

Probe ID	EST Accession #	Protein Name	Source	p-value
Development				
HAC01518	CN854114	BMI1 polycomb ring finger oncogene	Homo sapiens	0.01
HAZ15352_1	FD584612	Cuticle protein 6	BcNCP14.9	0.02
HAZ09065_1	EH117289	Cuticle protein CUT5	Portunus pelagicus	0.01
HAZ15734_1	FD585338	Cuticle protein CUT8	Portunus pelagicus	0.01
HAZ07638_1	EX471158	DEAD box ATP-dependent RNA helicase	Culex quinquefasciatus	0.01
HAZ13093_1	EY290967	Egalitarian CG4051-PA isoform 1	Apis mellifera	0.01
HAZ17904_1	FE841376	Myosin binding subunit CG32156-PE, isoform E	Drosophila melanogaster	0.02
HAZ17190_1	FE659382	Slow muscle myosin S1 heavy chain	Homarus americanus	0.01
HAZ06380_1	DV774644	Sparc	Schistocerca gregaria	0.01
HAZ_CL365_1	FF277784	Tubulin beta-2 chain	N/A	0.01
Metabolism				
HAC03749	FE841192	Aminoadipate aminotransferase	Bos taurus	0.02
HAZ08701_1	EH116731	Aminopeptidase N 2	Lymantria dispar	0.02
HAZ17221_1	FE659432	Carbonic anhydrase	Tribolium castaneum	0.01
HAZ15647_1	FD585179	Carboxylesterase	Spodoptera litura	0.01
HAC03226	FD467433	CG32230 CG32230-PA, isoform A	Drosophila melanogaster	0.01
HAZ07830_1	EH035271	Cytochrome c oxidase polypeptide VIII	Drosophila mauritiana	0.02
HAZ16719_1	FE044290	Cytochrome c oxidase subunit Va	Argas monolakensis	0.02
HAZ05159_1	EX471548	Enolase (2-phosphoglycerate dehydratase) (2-phospho-D-glycerate hydro-lyase)	N/A	0.03
HAZ05841_1	GO271518	F1-ATP synthase beta subunit	Pacifastacus leniusculus	0.01
HAC00222	CN852678	Fructose-1,6-bisphosphatase 1	N/A	0.01
HAZ11363_1	FF277546	Hydrogen-transporting ATP synthase, G-subunit	Tribolium castaneum	0.02

HAZ07869_1	EH035331	Intracellular protein transport protein USO1	Entamoeba dispar	0.01
HAZ17566_1	FE660161	Kexokinase	Litopenaeus vannamei	0.01
HAZ06126_1	FF277386	Lactate dehydrogenase	Brugia malayi	0.02
HAZ04277_1	DV774590	Masquerade-like protein	Pacifastacus leniusculus	0.02
HAZ04123_1	FE659845	NADH dehydrogenase subunit 2	Pseudocarcinus gigas	0.02
HAC00648	FE840896	NADH dehydrogenase subunit 4	Shinkaia crosnieri	0.03
HAZ17309_1	FE659606	Oxysterol binding protein-like 1A	Danio rerio	0.04
HAZ_CL133_1	EX471548	Phosphopyruvate hydratase	Penaeus monodon	0.01
HAZ14712_1	FD468128	Williams Beuren syndrome chromosome region 22	Culex quinquefasciatus	0.01
Immune & Oxidative Stress Response				
HAZ05970_1	DV774869	Crustin-like peptide type 4	Marsupenaeus japonicus	0.01
HAZ17835_1	FE841255	DD5	Marsupenaeus japonicus	0.01
HAZ03736_1	CN952197	α-2-macroglobulin	Macrobrachium rosenbergii	0.01
HAZ10635_1	FE043774	Kazal proteinase inhibitor	Biomphalaria glabrata	0.01
HAZ17687_1	FE840986	Serine protease	Aedes aegypti	0.03
HAZ05852_1	DV773884	Epsilon subunit of ATP synthetase	Hydroides elegans	0.02
HAZ04407_1	FE043609	Glutathione peroxidase	Metapenaeus ensis	0.01
HAC00663	EH116286	Glutathione S-transferase M	Danio rerio	0.02
HAZ07479_1	EH034769	MutY homolog	Monodelphis domestica	0.02
HAZ10357_1	EW997855	Oxidoreductase	Culex quinquefasciatus	0.01
HAZ04403_1	DV771759	Peroxinectin	Pacifastacus leniusculus	0.03
HAC00882	FD467404	Sulfotransferase (sult)	Aedes aegypti	0.01
Transcription/Translation				
HAZ11503_1	EX487309	Ecdysone response nuclear receptor FTZ-F1	Tribolium castaneum	0.01
HAZ12847_1	FF277333	mRNA capping enzyme	Tribolium castaneum	0.05
HAZ12696_1	FD699271	MAPK activating protein PM28 isoform 3	Macaca mulatta	0.04

HAZ13004_1	EY290805	DEAD box ATP-dependent RNA helicase	Nasonia vitripennis	0.01
HAC03276	CN951633	Farnesoic acid o-methyltransferase	Homarus americanus	0.01
HAC03095	CN951404	GIY-YIG domain containing 2	Acyrthosiphon pisum	0.01
HAC02295	FC071832	Histone h2a	Aedes aegypti	0.02
HAZ08952_1	FE044098	Lipoyltransferase 1	Bos taurus	0.04
HAZ10161_1	FC556532	Origin recognition complex, subunit 2-like	Equus caballus	0.03
HAC00272	FF277116	Polymerase (DNA directed), alpha 2 (70kD subunit)	Strongylocentrotus purpuratus	0.01
HAC03706	CN952204	Protein BUD31 homolog	Acyrthosiphon pisum	0.01
HAZ09452_1	EH401833	TAF6-like RNA polymerase II, p300\CBP-associated factor (PCAF)-associated factor	Strongylocentrotus purpuratus	0.02
HAC02461	EH116158	Transglutaminase	Acyrthosiphon pisum	0.04
HAZ17216_1	FE659426	tRNA isopentenyltransferase 1	Tribolium castaneum	0.01
HAC01650	CN854267	Eukaryotic translation initiation factor 2B, subunit 2 beta	Equus caballus	0.01
HAZ08103_1	EH035677	Hypothetical protein	Tribolium castaneum	0.01
HAZ14697_1	FD468099	Hypothetical protein AaeL_AAEL000427	Aedes aegypti	0.01
HAZ12518_1	EY116790	Hypothetical protein CBG22019	Caenorhabditis briggsae	0.01
HAZ07732_1	FE535075	Processing of precursor 7, ribonuclease P family	Danio rerio	0.01
HAZ04446_1	DV771818	Tubulin cofactor a	Rattus norvegicus	0.04
HAZ16336_1	FE043527	Histone H1	Anopheles stephensi	0.01
Other Genes				
Annotated: 41 Unannotated: 151				$p<0.05$